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PATENT

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Applicant:	Karl E. Giersckey et al.	Examiner:	Unknown
Serial No.:	Unknown	Group Art Unit:	Unknown
Filed:	Concurrently herewith	Docket:	697.002US1
Title:	ESTERS OF 5-AMINOLEVULINIC ACID AS PHOTSENSITIZING AGENTS IN PHOTOCHEMOTHERAPY		

PRELIMINARY AMENDMENT

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Before taking up the above-identified U.S. national phase patent application for examination, please enter the amended sheets for pages 1, 3-9, 9a, 11, and 13-16 (amended description) and the amended sheets for pages 36-39 (amended claims 1 through 18) that are attached to the International Preliminary Examination Report dated June 24, 1997.

Please further amend the claims as follows:

In claim 5, line 1, please delete "any one of claims 2 to 4" and insert therefor
--claim 2--.

In claim 6, line 1, please delete "any one of claims 2 to 5" and insert therefor
--claim 2--.

In claim 7, line 2, please delete "any one of claims 1 to 6" and insert therefor
--claim 2--.

In claim 9, line 1, please delete "or 8".

In claim 10, line 2, please delete "any one of claims 1 to 6" and insert therefor
--claims 1 or 2--.

In claim 11, lines 1-2, please delete "any one of claims 1 to 6" and insert therefor
--claims 1 or 2--.

In claim 15, lines 1-2, please delete "any one of claims 1 to 6" and insert therefor
--claims 1 or 2--.

In claim 17, lines 6-7, please delete "any one of claims 1 to 6" and insert therefor
--claims 1 or 2--.

In claim 18, line 5, please delete "any one of claims 1 to 6" and insert therefor
--claims 1 or 2--.

Please add the following new claims:

19. A process for preparing the compounds as defined in claim 1, comprising forming an ester of the carboxy group of a 5-aminolevulinic acid.

20. A process as claimed in claim 19, comprising reacting a 5-aminolevulinic acid, or an esterifiable derivative thereof, with an alcohol or an ester-forming derivative thereof.

Please enter the above-described amendments before taking up the application for examination.


Respectfully submitted,

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Matthew Hollister
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62655/002.588

Esters of 5-aminolevulinic acid as
Photosensitizing Agents in Photochemotherapy

The present invention relates to derivatives of 5-aminolevulinic acid (ALA) and in particular to esters of ALA for use as photosensitizing agents in photochemotherapy or diagnosis.

Photochemotherapy, or photodynamic therapy (PDT) as it is also known, is a recently up-coming technique for the treatment of various abnormalities or disorders of the skin or other epithelial organs or mucosa, especially cancers or pre-cancerous lesions, as well as certain non-malignant lesions for example skin complaints such as psoriasis. Photochemotherapy involves the application of photosensitizing (photochemotherapeutic) agents to the affected area of the body, followed by exposure to photoactivating light in order to activate the photosensitizing agents and convert them into cytotoxic form, whereby the affected cells are killed or their proliferative potential diminished.

A range of photosensitizing agents are known, including notably the psoralens, the porphyrins, the chlorins and the phthalocyanins. Such drugs become toxic when exposed to light.

Photosensitizing drugs may exert their effects by a variety of mechanisms, directly or indirectly. Thus for example, certain photosensitizers become directly toxic when activated by light, whereas others act to generate toxic species, e.g. oxidising agents such as singlet oxygen or other oxygen-derived free radicals, which are extremely destructive to cellular material and biomolecules such as lipids, proteins and nucleic acids. Psoralens are an example of directly acting photosensitizers; upon exposure to light they form adducts and cross-links between the two strands of DNA

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molecules, thereby inhibiting DNA synthesis. The unfortunate risk with this therapy is that unwanted mutagenic and carcinogenic side effects may occur.

This disadvantage may be avoided by selecting photosensitizers with an alternative, indirect mode of action. For example porphyrins, which act indirectly by generation of toxic oxygen species, have no mutagenic side effects and represent more favourable candidates for photochemotherapy. Porphyrins are naturally occurring precursors in the synthesis of heme. In particular, heme is produced when iron (Fe^{3+}) is incorporated in protoporphyrin IX (Pp) by the action of the enzyme ferrochelatase. Pp is an extremely potent photosensitizer, whereas heme has no photosensitizing effect.

One such porphyrin-based drug, Photofrin, has recently been approved as a photosensitizer in the therapy of certain cancers. The main disadvantage is that since it must be administered parenterally, generally intravenously, cause photosensitization of the skin which may last for several weeks following i.v. injection. Photofrin consists of large oligomers of porphyrin and it does not readily penetrate the skin when applied topically. Similar problems exist with other porphyrin-based photosensitizers such as the so-called "hematoporphyrin derivative" (HpD) which has also been reported for use in cancer photochemotherapy (see for example S. Dougherty, J. Natl. Cancer Ins., 1974, 52; 1333; Kelly and Snell, J. Urol, 1976, 115 : 150). HpD is a complex mixture obtained by treating haematoporphyrin with acetic and sulphuric acids, after which the acetylated product is dissolved with alkali.

To overcome these problems, precursors of Pp have been investigated for photochemotherapeutic potential. In particular the Pp precursor 5-aminolevulinic acid (ALA) has been investigated as a photochemotherapeutic agent for certain skin cancers. ALA, which is formed

from succinyl CoA and glycine in the first step of heme synthesis, is to a limited extent able to penetrate the skin and lead to a localised build-up of Pp; since the action of ferrochelatase (the metallating enzyme) is the rate limiting step in heme synthesis, an excess of ALA leads to accumulation of Pp, the photosensitizing agent. Thus, by applying ALA topically to skin tumours, and then after several hours exposing the tumours to light, a beneficial photochemotherapeutic effect may be obtained (see for example WO91/01727). Since the skin covering basilomas and squamous cell carcinomas is more readily penetrated by ALA than healthy skin, and since the concentration of ferrochelatase is low in skin tumours, it has been found that topical application of ALA leads to a selectively enhanced production of Pp in tumours.

However, whilst the use of ALA represents a significant advance in the art, photochemotherapy with ALA is not always entirely satisfactory. ALA is not able to penetrate all tumours and other tissues with sufficient efficacy to enable treatment of a wide range of tumours or other conditions and ALA also tends to be unstable in pharmaceutical formulations. A need therefore exists for improved photochemotherapeutic agents.

The present invention addresses this need and in particular aims to provide photochemotherapeutic agents which are better able to penetrate the tumour or other abnormality, and which have an enhanced photochemotherapeutic effect over those described in the prior art.

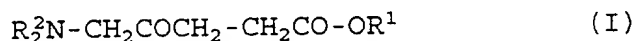
In one aspect, the present invention thus provides compounds being esters of 5-aminolevulinic acids or pharmaceutically acceptable salts thereof for use in photochemotherapy or diagnosis.

In the esters of the invention the 5-amino group may be substituted or unsubstituted, the latter case being the ALA esters.

More particularly, the compounds for use according to the invention are esters of 5-aminolevulinic acids with optionally substituted alkanols, ie. alkyl esters or substituted alkyl esters.

Database Xfire, entries 3060978, 5347132, 5499790, 5620924, 5633390, 5991317 and 6517740 (Beilstein); Cosmo Sogo Kenkyusho KK, Patent Abstracts of Japan, Vol 16; No. 156 (C-0930), 16.4.1992; EP-A-316179 (Tokuyama Soda KK); GB-A-2058077 (Hudson et al) and DE-A-2411382 (Boehringer Sohn Ingelheim) describe alkyl ester derivative of 5-aminolevulinic acid, and derivatives and salts thereof and processes for their preparation.

Alternatively viewed, the invention can therefore be seen to provide compounds of formula I,



(wherein R^1 may represent alkyl optionally substituted by hydroxy, alkoxy, acyloxy, alkoxy carbonyloxy, amino, aryl, oxo or fluoro groups and optionally interrupted by oxygen, nitrogen, sulphur or phosphorus atoms; and R^2 , each of which may be the same or different, represents a hydrogen atom or a group R^1) and salts thereof for use in photochemotherapy or diagnosis.

The substituted alkyl R^1 groups may be mono or poly-substituted. Thus suitable R^1 groups include for example unsubstituted alkyl, alkoxyalkyl, hydroxyalkoxyalkyl, polyhydroxyalkyl, hydroxy poly alkyleneoxyalkyl and the like. The term "acyl" as used herein includes both carboxylate and carbonate groups, thus, acyloxy substituted alkyl groups include for example alkylcarbonyloxy alkyl. In such groups any alkylene moieties preferably have carbon atom contents defined for alkyl groups below. Preferred aryl groups include

phenyl and monocyclic 5-7 membered heteroaromatics, especially phenyl and such groups may themselves optionally be substituted.

Representative substituted alkyl groups R^1 include alkoxymethyl, alkoxyethyl and alkoxypropyl groups or acyloxymethyl, acyloxyethyl and acyloxypropyl groups eg. pivaloyloxymethyl.

Preferred compounds for use according to the invention, include those wherein R^1 represents an unsubstituted alkyl group and/or each R^2 represents a hydrogen atom.

As used herein, the term "alkyl" includes any long or short chain, straight-chained or branched aliphatic saturated or unsaturated hydrocarbon group. The unsaturated alkyl groups may be mono- or polyunsaturated and include both alkenyl and alkynyl groups. Such groups may contain up to 40 carbon atoms. However, alkyl groups containing up to 10 eg. 8, more preferably up to 6, and especially preferably up to 4 carbon atoms are preferred.

Particular mention may be made of ALA-methylester, ALA-ethylester, ALA-propylester, ALA-hexylester, ALA-heptylester and ALA-octylester and salts thereof, which represent preferred compounds for use according to the invention.

The compounds for use in the invention may be prepared using standard processes and procedures well-known in the art for derivatization of multi-functional compounds, and especially esterification. As known in the art, such esterification of compounds may involve protection and deprotection of appropriate groups such that only the required groups remain active and take part in the reaction under the conditions of the esterification. Thus for example the substituents of substituted alkanols used to prepare the esters may be protected during esterification. Similarly the NR_2^2 group on the compound contributing this group to

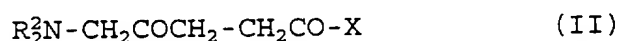
compounds of formula I may be protected during the reaction and deprotected thereafter. Such protection/deprotection procedures are well known in the art for the preparation of derivatives, and in particular, esters of well known amino-acids, see for example Mcomie in "Protective Groups in Organic Chemistry", Plenum, 1973 and T.W. Greene in "Protective Groups in Organic Chemistry", Wiley-Interscience, 1981.

In a further aspect, the present invention thus provides a process for preparing the compounds for use in the invention, comprising forming an ester of the carboxy group of a 5-aminolevulinic acid.

The invention can thus be seen to provide a process for preparing the compounds for use in the invention, comprising reacting a 5-aminolevulinic acid, or an esterifiable derivative thereof, with an alkanol or an ester-forming derivative thereof.

More particularly, this aspect of the invention provides a process for preparing compounds of formula I, which process comprises at least one of the following steps:

- (a) reacting a compound of formula II



(wherein X represents a leaving group, for example a hydroxyl group, a halogen atom or alkoxy group or COX represents an acid anhydride group and R^2 is as hereinbefore defined)

with a compound of formula III



(wherein R^1 is as hereinbefore defined); and

- (b) converting a compound of formula I into a

pharmaceutically acceptable salt thereof.

The reaction of step (a) may conveniently be carried out in a solvent or mixture of solvents such as water, acetone, diethylether, methylformamide, tetrahydrofuran etc. at temperatures up to the boiling point of the mixture, preferably at ambient temperatures.

The conditions of the esterification reactions will depend of the alcohol used and the conditions may be chosen such that maximum yield of the ester is obtained. Since the esterification reactions are reversible equilibrium reactions, reaction conditions may be selected in such a way that maximum yield of the ester product is obtained. Such conditions may be obtained by selecting a solvent which is capable of removing the water formed in a typical esterification reaction by forming an azeotrope with water. Such solvents are exemplified by aromatic hydrocarbons or others capable of forming azeotropes with water, e.g. some chlorinated hydrocarbons such as chloroform. For the formation of the lower esters of 5-ALA the equilibrium reaction may be driven to the ester side by using a large excess of the alcohol. With other esters the equilibrium may be driven towards the ester product by using a large excess of the acid.

Esterification reactions are well-known in the art for example, as described by Saul Patai in "The chemistry of the carboxylic acids and esters", (Ch. 11, p. 505, Interscience 1969) and Houban Weyl, (Methoden der Organische Chemie, Band E5, "Carbonsauren und carbonsauren-derivate", p. 504, Georg Thieme Verlag, 1985). The formation of derivatives of amino-acids are described in Band XI/2 of the same series, (Houben Weyl, Methoden der Organische Chemie, Band XI/2, "Stickstoffverbindungen", p. 269, Georg Thieme Verlag, 1958).

The reaction will conveniently be carried out in

the presence of a catalyst, eg. an inorganic or organic acid or an acid binding agent such as a base.

The compounds used as starting materials are known from the literature, and in many cases commercially available, or may be obtained using methods known per se. ALA, for example, is available from Sigma or from Photocure, Oslo, Norway.

As mentioned above, the compounds for use according to the invention may take the form of pharmaceutically acceptable salts. Such salts preferably are acid addition salts with physiologically acceptable organic or inorganic acids. Suitable acids include, for example, hydrochloric, hydrobromic, sulphuric, phosphoric, acetic, lactic, citric, tartaric, succinic, maleic, fumaric and ascorbic acids. Procedures for salt formation are conventional in the art.

As mentioned above, the compounds for use according to the invention and their salts have valuable pharmacological properties, namely a photosensitizing agent which renders them useful as photochemotherapeutic agents.

Like ALA, the compounds exert their effects by enhancing production of Pp; upon delivery to the desired site of action hydrolytic enzymes such as esterases present in the target cells break down the esters into the parent ALA, which then enters the haem synthesis pathway and leads to a build-up of Pp. However, the compounds for use according to the invention have a number of advantages over ALA itself. Firstly, the compounds are better able to penetrate skin and other tissues as compared with ALA; the penetration is both deeper and faster. This is an important advantage, especially for topical administration. Secondly, the esters have surprisingly been found to be better enhancers of Pp production than ALA; Pp production levels following administration of the ALA esters are higher than with ALA alone. Thirdly, the compounds for

use in the invention demonstrate improved selectivity for the target tissue to be treated, namely the Pp production-enhancing effect is localised to the desired target lesion and does not spread to the surrounding tissues. This is especially evident with tumours. Finally, the compounds appear to localise better to the target tissue upon administration. This is especially important for systemic application, since it means that undesirable photosensitization effects, as reported in the literature for other porphyrin-based photosensitizers, may be reduced or avoided.

A further aspect of the present invention accordingly provides a pharmaceutical composition comprising a compound as described hereinbefore, or a pharmaceutically acceptable salt thereof, together with at least one pharmaceutical carrier or excipient.

In a still further aspect, there is also provided the use of a compound as described hereinbefore, or a pharmaceutically acceptable salt thereof, for the preparation of a therapeutic agent for use in photochemotherapy, and especially for the treatment of disorders or abnormalities of external or internal surfaces of the body which are responsive to photochemotherapy.

The abnormalities and disorders which may be treated according to the present invention include any malignant, pre-malignant and non-malignant abnormalities or disorders responsive to photochemotherapy eg. tumours or other growths, skin disorders such as psoriasis or actinic keratoses, skin abrasions, and other diseases or infections eg. bacterial, viral or fungal infections, for example Herpes virus infections. The invention is particularly suited to the treatment of diseases, disorders or abnormalities where discrete lesions are formed to which the compositions may be directly applied (lesions is used here in a broad sense to include tumours and the like).

The internal and external body surfaces which may be treated according to the invention include the skin and all other epithelial and serosal surfaces, including for example mucosa, the linings of organs eg. the respiratory, gastro-intestinal and genito-urinary tracts, and glands with ducts which empty onto such surfaces (e.g. liver, hair follicles with sebaceous glands, mammary glands, salivary glands and seminal vesicles). In addition to the skin, such surfaces include for example the lining of the vagina, the endometrium and the urothelium. Such surfaces may also include cavities formed in the body following excision

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of diseased or cancerous tissue eg. brain cavities following the excision of tumours such as gliomas.

Exemplary surfaces thus include: (i) skin and conjunctiva; (ii) the lining of the mouth, pharynx, oesophagus, stomach, intestines and intestinal appendages, rectum, and anal canal; (iii) the lining of the nasal passages, nasal sinuses, nasopharynx, trachea, bronchi, and bronchioles; (iv) the lining of the ureters, urinary bladder, and urethra; (v) the lining of the vagina, uterine cervix, and uterus; (vi) the parietal and visceral pleura; (vii) the lining of the peritoneal and pelvic cavities, and the surface of the organs contained within those cavities; (viii) the dura mater and meninges; (ix) any tumors in solid tissues that can be made accessible to photoactivating light e.g. either directly, at time of surgery, or via an optical fibre inserted through a needle.

The compositions of the invention may be formulated in conventional manner with one or more physiologically acceptable carriers or excipients, according to techniques well known in the art. Compositions may be administered topically, orally or systemically. Topical compositions are preferred, and include gels, creams, ointments, sprays, lotions, salves, sticks, soaps, powders, pessaries, aerosols, drops and any of the other conventional pharmaceutical forms in the art.

Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will, in general, also contain one or more emulsifying, dispersing, suspending, thickening or colouring agents. Powders may be formed with the aid of any suitable powder base. Drops may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing, solubilising or suspending agents. Aerosol sprays are conveniently delivered from

pressurised packs, with the use of a suitable propellant.

Alternatively, the compositions may be provided in a form adapted for oral or parenteral administration, for example by intradermal, subcutaneous, intraperitoneal or intravenous injection. Alternative pharmaceutical forms thus include plain or coated tablets, capsules, suspensions and solutions containing the active component optionally together with one or more inert conventional carriers and/or diluents, e.g. with corn starch, lactose, sucrose, microcrystalline cellulose, magnesium stearate, polyvinylpyrrolidone, citric acid, tartaric acid, water, water/ethanol, water/glycerol, water/sorbitol, water/polyethyleneglycol, propyleneglycol, stearylalcohol, carboxymethylcellulose or fatty substances such as hard fat or suitable mixtures thereof.

The concentration of the compounds as described hereinbefore in the compositions, depends upon the nature of the compound, the composition, mode of administration and the patient and may be varied or adjusted according to choice. Generally however, concentration ranges of 1 to 50% (w/w) are suitable. For therapeutic applications concentration ranges of 10 to 50% have been found to be suitable, eg. 15 to 30% (w/w).

Following administration to the surface, the area treated is exposed to light to achieve the photo-chemotherapeutic effect. The length of time following administration, at which the light exposure takes place will depend on the nature of the composition and the form of administration. This can generally be in the order of 0.5 to 48 hours, e.g. 1 to 10 hours.

The irradiation will in general be applied at a dose level of 40 to 200 Joules/cm², for example at 100 Joules/cm².

The wavelength of light used for irradiation may be selected to achieve a more efficacious photochemo-

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therapeutic effect. Conventionally, when porphyrins are used in photochemotherapy they are irradiated with light at about the absorption maximum of the porphyrin. Thus, for example in the case of the prior art use of ALA in photochemotherapy of skin cancer, wavelengths in the region 350-640 nm, preferably 610-635 nm were employed. However, by selecting a broad range of wavelengths for irradiation, extending beyond the absorption maximum of the porphyrin, the photosensitizing effect may be enhanced. Whilst not wishing to be bound by theory, this is thought to be due to the fact that when Pp, and other porphyrins, are exposed to light having wavelengths within its absorption spectrum, it is degraded into various photo-products including in particular photoporphyrin (PPp). PPp is a chlorin and has a considerable photo-sensitizing effect; its absorption spectrum stretches out to longer wavelengths beyond the wavelengths at which Pp absorbs ie. up to almost 700 nm (Pp absorbs almost no light above 650 nm). Thus in conventional photochemotherapy, the wavelengths used do not excite PPp and hence do not obtain the benefit of its additional photosensitizing effect. Irradiation with wavelengths of light in the range 500-700 nm has been found to be particularly effective. It is particularly important to include the wavelengths 630 and 690 nm.

A further aspect of the invention thus provides a method of photochemotherapeutic treatment of disorders or abnormalities of external or internal surfaces of the body, comprising administering to the affected surfaces, a composition as hereinbefore defined, and exposing said surfaces to light, preferably to light in the wavelength region 300-800 nm, for example 500-700 nm.

Methods for irradiation of different areas of the body, eg. by lamps or lasers are well known in the art (see for example Van den Bergh, Chemistry in Britain, May 1986 p. 430-439).

The compounds for use in the invention may be formulated and/or administered with other photosensitizing agents, for example ALA or photofrin, or with other active components which may enhance the photochemotherapeutic effect. For example, chelating agents may beneficially be included in order to enhance accumulation of Pp; the chelation of iron by the chelating agents prevents its incorporation into Pp to form haem by the action of the enzyme ferrochelatase, thereby leading to a build-up of Pp. The photosensitizing effect is thus enhanced.

Aminopolycarboxylic acid chelating agents are particularly suitable for use in this regard, including any of the chelants described in the literature for metal detoxification or for the chelation of paramagnetic metal ions in magnetic resonance imaging contrast agents. Particular mention may be made of EDTA, CDTA (cyclohexane diamine tetraacetic acid), DTPA and DOTA. EDTA is preferred. To achieve the iron-chelating effect, desferrioxamine and other siderophores may also be used, e.g. in conjunction with aminopolycarboxylic acid chelating agents such as EDTA.

The chelating agent may conveniently be used at a concentration of 1 to 20% eg. 2 to 10% (w/w).

Additionally, it has been found that surface-penetration assisting agents and especially dialkylsulphoxides such as dimethylsulphoxide (DMSO) may have a beneficial effect in enhancing the photochemotherapeutic effect. This is described in detail in our co-pending application No. PCT/GB94/01951, a copy of the specification of which is appended hereto.

The surface-penetration assisting agent may be any of the skin-penetration assisting agents described in the pharmaceutical literature e.g. HPE -101 (available from Hisamitsu), DMSO and other dialkylsulphoxides, in particular n-decylmethyl-sulphoxide (NDMS), dimethylsulphacetamide, dimethylformamide (DMFA),

dimethylacetamide, glycols, various pyrrolidone derivatives (Woodford et al., J. Toxicol. Cut. & Ocular Toxicology, 1986, 5: 167-177), and Azone® (Stoughton et al., Drug Dpv. Ind. Pharm. 1983, 9: 725-744), or mixtures thereof.

DMSO however has a number of beneficial effects and is strongly preferred. Thus, in addition to the surface-penetration assisting effect (DMSO is particularly effective in enhancing the depth of penetration of the active agent into the tissue), DMSO has anti-histamine and anti-inflammatory activities. In addition, DMSO has been found to increase the activity of the enzymes ALA-synthase and ALA-dehydrogenase (the enzymes which, respectively, form and condense ALA to porphobilinogen) thereby enhancing the formation of the active form, Pp.

The surface penetration agent may conveniently be provided in a concentration range of 2 to 50% (w/w), eg about 10% (w/w).

According to the condition being treated, and the nature of the composition, the compounds for use in the invention may be co-administered with such other optional agents, for example in a single composition or they may be administered sequentially or separately. Indeed, in many cases a particularly beneficial photochemotherapeutic effect may be obtained by pre-treatment with the surface-penetration assisting agent in a separate step, prior to administration of the compounds for use in the invention. Furthermore, in some situations a pre-treatment with the surface-penetration assisting agent, followed by administration of the photochemotherapeutic agent in conjunction with the surface-penetration assisting agent may be beneficial. When a surface-penetration assisting agent is used in pre-treatment this may be used at high concentrations, e.g. up to 100% (w/w). If such a pre-treatment step is employed, the photochemotherapeutic

agent may subsequently be administered up to several hours following pre-treatment eg. at an interval of 5-60 minutes following pre-treatment.

Viewed from a further aspect, the invention thus provides a product comprising a compound as described hereinbefore or a pharmaceutically acceptable salt thereof, together with at least one surface-penetration assisting agent, and optionally one or more chelating agents as a combined preparation for simultaneous, separate or sequential use in treating disorders or abnormalities of external or internal surfaces of the body which are responsive to photochemotherapy.

Alternatively viewed, this aspect of the invention also provides a kit for use in photochemotherapy of disorders or abnormalities of external or internal surfaces of the body comprising:

- a) a first container containing a compound as described hereinbefore or a pharmaceutically acceptable salt thereof,
- b) a second container containing at least one surface penetration assisting agent; and optionally
- c) one or more chelating agents contained either within said first container or in a third container.

Where the surface penetration agent is applied in a separate pre-treatment step, it may be applied in higher concentration, for example up to 100% (w/w).

It will be appreciated that the method of therapy using compounds as described hereinbefore inevitably involves the fluorescence of the disorder or abnormality to be treated. Whilst the intensity of this fluorescence may be used to eliminate abnormal cells, the localization of the fluorescence may be used to visualize the size, extent and situation of the abnormality or disorder. This is made possible through

the surprising ability of ALA esters to preferentially localize to non-normal tissue.

The abnormality or disorder thus identified or confirmed at the site of investigation may then be treated through alternative therapeutic techniques e.g. surgical or chemical treatment, or by the method of therapy of the invention by continued build up of fluorescence or through further application of compounds of the invention at the appropriate site. It will be appreciated that diagnostic techniques may require lower levels of fluorescence for visualization than used in therapeutic treatments. Thus, generally, concentration ranges of 1 to 50% e.g. 1-5% (w/w) are suitable. Sites, methods and modes of administration have been considered before with regard to the therapeutic uses and are applicable also to diagnostic uses described here. The compounds for use in the invention may also be used for in vitro diagnostic techniques, for example for examination of the cells contained in body fluids. The higher fluorescence associated with non-normal tissue may conveniently be indicative of an abnormality or disorder. This method is highly sensitive and may be used for early detection of abnormalities or disorders, for example bladder or lung carcinoma by examination of the epithelial cells in urine or sputum samples, respectively. Other useful body fluids which may be used for diagnosis in addition to urine and sputum include blood, semen, tears, spinal fluid etc. Tissue samples or preparations may also be evaluated, for example biopsy tissue or bone marrow samples. The present invention thus extends to the use of compounds of the invention, or salts thereof for diagnosis according to the aforementioned methods for photochemotherapy, and products and kits for performing said diagnosis.

A further aspect of the invention relates to a method of in vitro diagnosis, of abnormalities or

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disorders by assaying a sample of body fluid or tissue of a patient, said method comprising at least the following steps:

- i) admixing said body fluid or tissue with a compound as described hereinbefore,
- ii) exposing said mixture to light,
- iii) ascertaining the level of fluorescence, and
- iv) comparing the level of fluorescence to control levels.

The invention will now be described in more detail in the following non-limiting Examples, with reference to the drawings in which:

Figure 1 shows fluorescence intensity (relative units vs wavelength (nm)) of PpIX in the normal skin of mice after topical administration of

- (A) free ALA
- (B) ALA methylester
- (C) ALA ethylester
- (D) ALA propylester

after 0.5, 1, 1.5, 2.5, 3, 3.5 and 14 hours following administration;

Figure 2 shows the distribution of PpIX as measured by fluorescence intensity (relative units vs wavelength (nm)) in Brain, dermis, Ear, Liver and muscle 14 hours after topical administration to the normal skin of mice:

- (A) free ALA
- (B) ALA methylester
- (C) ALA ethylester
- (D) ALA propylester;

Figure 3 shows PpIX fluorescence (fluorescence intensity, relative units vs wavelength (nm)) in the skin of mice

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15 minutes, 1 hour, 4 hours and 10 hours after intraperitoneal injection of ALA methylester (150 mg/kg);

Figure 4 shows PpIX fluorescence (fluorescence intensity relative units vs wavelength (nm)) (A) 1.5 hours and (B) 4 hours after topical administration of ALA methylester to basal cell carcinoma (BCC) lesions on the skin of human patients (- tumour; --- normal skin);

Figure 5 shows PpIX fluorescence (fluorescence intensity relative units vs wavelength (nm)) (A) 1.5 hours and (B) 4 hours after topical administration of ALA ethylester to basal cell carcinoma (BCC) lesions on the skin of human patients (- tumour; --- normal skin);

Figure 6 shows PpIX fluorescence (fluorescence intensity relative units vs wavelength (nm)) (A) 1.5 hours and (B) 4 hours after topical administration of ALA propylester to basal cell carcinoma (BCC) lesions on the skin of human patients (- tumour; --- normal skin);

Figure 7 shows PpIX fluorescence (fluorescence intensity relative units vs wavelength (nm)) (A) 1.5 hours and (B) 4 hours after topical administration of ALA to basal cell carcinoma (BCC) lesions on the skin of human patients (- tumour; --- normal skin);

Figure 8 shows measurement of PpIX production following topical application of ALA methylester in human BCC and surrounding normal skin by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 9 shows measurement of PpIX production following topical application of ALA in human BCC and surrounding

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normal skin by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 10 shows PpIX fluorescence (fluorescence intensity relative units vs wavelength (nm)) 24 hours following topical administration of ALA methylester to BCC lesion and to normal skin of human patients.

Figure 11 shows PpIX fluorescence (fluorescence intensity relative units vs wavelength (nm)) 24 hours following topical administration of ALA to BCC lesion and to normal skin of human patients.

Figure 12 shows measurement of PpIX production 4.5 hours following topical application of ALA methylester in human BCC by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 13 shows measurement of PpIX production 4.5 hours following topical application of ALA methylester in human normal skin by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 14 shows measurement of PpIX production 24 hours following topical application of ALA methylester in human BCC by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 15 shows measurement of PpIX production 24 hours following topical application of ALA methylester in human normal skin by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

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Figure 16 shows measurement of PpIX production 24 hours following topical application of free ALA in human BCC by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 17 shows measurement of PpIX production 24 hours following topical application of free ALA in human normal skin by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 18 shows measurement of PpIX production 4.5 hours following topical application of free ALA and 20% DMSO in human BCC by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 19 shows measurement of PpIX production 4.5 hours following topical application of free ALA and 20% DMSO in human normal skin by CDD microscopy of biopsies (A) graphical representation showing fluorescence intensity vs depth (μm) and (B) micrograph;

Figure 20 shows a time course (fluorescence intensity relative units vs time (hours)) of ALA methylester-induced (PpIX) fluorescence in the mouse skin after topical application of ALA methylester alone (-●-), ALA methylester plus DMSO (-▲-), ALA methylester plus desferrioxamine (DF) (-■-) or ALA methylester plus DF and DMSO (-▼-). Each point is the mean of measurements from at least three mice;

Figure 21 shows fluorescence photographs of the mouse skin taken 1 h after topical application of free ALA alone (A), ALA methylester (B), ALA ethylester (C) and ALA propylester (D), showing fluorescence in the

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epidermis (Ep), epithelial hair follicles and sebaceous gland (arrows), but not in the dermis (De). Original magnification x250.

Figure 22 is a graph showing relative tumour volume against time (days) following treatment of WiDr human colonic carcinoma transplanted subcutaneously into nude mice with ALA or ALA methylester plus DF; (-▲-) control; (-▼-) DF alone; (-■-) ALA + DF + DMSO; (-●-) ALA methylester + DF + DMSO.

Figure 23 shows PpIX fluorescence ratios between BCC lesions and surrounding normal skin after topical application of ALA or its esters.

Example 1

Preparation of methyl 5-aminolevulinate hydrochloride

To a 500 ml glass reactor containing 200 ml methanol, was added 1g 5-amino-levulinic acid hydrochloride and 1 drop conc. HCl. The reaction mixture was then stirred overnight at 60°C. The progress of the esterification was followed by ¹H-NMR. Excess methanol was removed by distillation, and the product further dried under vacuum at 30-40°C, giving methyl 5-aminolevulinate hydrochloride. The structure was confirmed by ¹H-NMR in DMSO-d₆.

Example 2

Preparation of ethyl 5-aminolevulinate hydrochloride (ALA ethylester)

1g 5-aminolevulinic acid hydrochloride was added to 200 ml dry ethanol containing 1-2 drops conc. hydrochloric acid in a 250 ml glass reactor equipped with a stirrer, reflux condenser and a thermometer. The esterification was performed at reflux overnight (70-80°C). After the

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reaction had gone to completion, the ethanol was removed under vacuum. Finally, the product was dried under high vacuum at 30-40°C, giving 0.94g Ethyl 5-aminolevulinate hydrochloride. Confirmation of the structure was done by $^1\text{H-NMR}$ in DMSO-d_6 .

Example 3

Preparation of n-propyl 5-aminolevulinate hydrochloride (ALA propylester)

0.5g 5-aminolevulinic acid hydrochloride was dissolved in 100 ml dry n-propanol containing 1-2 drops of conc. hydrochloride in a 250 ml glass reactor equipped with a stirrer, reflux condenser and a thermometer. The reaction mixture was stirred at 70-80°C for approx. 20 hours. After all the 5-aminolevulinic acid was converted to its n-propylester (followed by $^1\text{H-NMR}$), the excess propanol was removed, and the product dried under high vacuum (<1 mBar) at 40-50°C. The reaction gave 0.49g propyl 5-aminolevulinate hydrochloride. The structure was confirmed by $^1\text{H-NMR}$ in DMSO-d_6 .

Example 4

Preparation of n-hexyl 5-aminolevulinic hydrochloride (ALA hexylester)

2 grams of 5-aminolevulinic acid hydrochloride was dissolved in 25 grams of dry n-hexanol with 5-6 drops of conc. hydrochloride added in a 50 ml glass reactor equipped with a reflux condenser and a thermometer. The reaction mixture was held at 50-60°C for approx. 3 days. The excess n-hexanol was removed under vacuum and the product finally dried under high vacuum, giving 2.4 grams of n-hexyl 5-aminolevulinate hydrochloride. The structure was confirmed by $^1\text{H-NMR}$ spectroscopy in DMSO-d_6 .

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Example 5Preparation of n-heptyl 5-aminolevulinic hydrochloride (ALA heptylester)

0.5g 5-aminolevulinic acid hydrochloride was added to 30 grams of n-heptanol containing 5 drops of conc. hydrochloride in a 100 ml glass reactor equipped with a stirrer, reflux condenser and a thermometer. After all the 5-aminolevulinic acid had dissolved, the reaction mixture was stirred at 70-80°C for approx. 48 hours. After the 5-aminolevulinic acid was converted to its n-heptyl ester (followed by ¹H-NMR), the excess alcohol was removed, and the product dried under high vacuum (< 1 mBar) at 70°C. The reaction gave 1.5g n-heptyl 5-aminolevulinate hydrochloride. The structure was confirmed by ¹H-NMR in DMSO-d₆.

Example 6Preparation of n-octyl 5-aminolevulinic hydrochloride (ALA octylester)

1 gram 5-aminolevulinic acid hydrochloride was added to 30 grams of dry n-octanol containing 5-6 drops of conc. hydrochloride in a 50 ml glass reactor equipped with a reflux condenser, stirrer and a thermometer. The reaction mixture was stirred at 65-70°C for approx. 2 days. Excess n-octanol was removed under vacuum and the product finally dried under high vacuum, giving 1.5 grams of n-octyl 5-aminolevulinate hydrochloride. The structure was confirmed by ¹H-NMR spectroscopy in DMSO-d₆.

Example 7Formulation

20% creams were prepared by admixture of the active component, ALA, ALA methylester, ALA ethylester, or ALA

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propylester (prepared according to Examples 1 to 3 respectively), with "Urguentum Merck" cream base (available from Merck) consisting of silicon dioxide, paraffin liq., vaseline, album, cetostearol., polysorbat. 40, glycerol monostearate, Miglyol®812 (a mixture of plant fatty acids), polypropyleneglycol., and purified water.

Corresponding creams were also prepared, additionally containing 3-20% DMSO.

Example 8

Determination of protoporphyrin IX production in the skin of mice by CCD microscopy of biopsies:

A commercial oil-in-water cream containing (20% w/w) one of the chemicals (free ALA, ALA methylester, ALA ethylester and ALA propylester) (see Example 1) was topically applied to the normal skin of nu/nu nude mice for 0.5, 1, 3 and 6 hours, then biopsied and evaluated by means of microscopic fluorescence photometry incorporating a light-sensitive thermol-electrically cooled charge coupled device (CCD) camera. The results show that free ALA and its three ester derivatives are taken up by the skin tissue, the esterified ALA derivatives are being deesterified in the skin, and converted into protoporphyrin IX (PpIX) 0.5 hours after topical application. The fluorescence intensity of PpIX in the skin increased with the time of the application and the maximum amounts of the fluorescence were seen about 6 hours (the latest time point studied) after the application in all cases.

Example 9

Measurements in situ of protoporphyrin IX production in the skin of mice by an optical-fiber based system

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The aim of this study was to investigate the build-up of esterified ALA ester-induced porphyrins fluorescence in the normal skin of nude mice in vivo after topical or systemic administration of ALA ester derivatives.

MATERIALS AND METHODS

Chemicals. 5-aminolevulinic acid (ALA) methyl-, ethyl- and propyl-esters ($\text{H}_2\text{N}-\text{CH}_2\text{COOCH}_2-\text{CH}_2\text{COO}-\text{R}$; R can be CH_3 , $\text{CH}_2-\text{CH}_2-\text{CH}_3$) were prepared by Norsk Hydro Research Center (Porsgrunn, Norway) as described in Examples 1 to 3. Free ALA hydrochloride and desferrioxamine mesylate (DF) were purchased from Sigma Chemical Company (St. Louis, Mo, USA). Dimethyl sulphoxide (DMSO) was obtained from Janssen Chimica (Geel, Belgium). Commercial oil-water creams (Unguentum Merck, Darmstadt, Germany) containing 20% one of the ALA ester derivatives (w/w), 20% free ALA, 20% ALA methylester plus 5% DF, 20% ALA methylester plus 20% DMSO, or 20% ALA methylester plus 5% DF and 20% DMSO were freshly prepared prior to use. All creams were made by the Pharmacy at the Norwegian radium Hospital. For intraperitoneal injection, ALA and its methylester were freshly dissolved in saline. All other chemicals used were of the highest purity commercially available.

Animals. Female Balb/c nu/nu athymic nude mice were obtained from the Animal Laboratory at the Norwegian Radium Hospital and kept under specific-pathogen-free conditions. At the start of the experiments the mice were 6-7 weeks old weighing 18-24 g. Three mice were housed per cage with autoclaved covers in a dark room during the experiments.

Treatment procedure. One of the creams was painted on the normal skin at right flank region of each mouse, and covered by a semi-permeable dressing (3M, St Paul, MN,

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USA) for various time intervals (from 0.25 to 24 h) before fluorescence measurements in situ or being biopsied for microscopic fluorescence imaging. About 0.2g cream was applied to an approximate 2.25 cm² area of the skin. In the case of i.p. injection the mice were given ALA or its methylester at a dose of 150 mg/kg. At least three mice were used for each condition.

Fluorescence spectroscopic measurements in situ. A perkin Elmer LS-50 fluorescence spectrometer equipped with a red-sensitive photomultiplier (Hamamatsu R 928) was used. This instrument has a pulsed Xenon arc light source and phase sensitive detection, such that fluorescence can be readily measured. Part of the excitation beam (set at 408 nm for fluorescence measurements) was reflected into a 600 μ m core multimodus optical quartz fiber (No. 3501 393, Dornier Medizintechnik, GmbH, Germering, Germany) by means of a mirror for application onto the subject through a hand held probe. Emission in the region of 550-750 nm was measured via emission fibres collecting information through the probe.

Fluorescence microscopy. After the creams were topically applied to the skin of mice for various times (as indicated above), the skin was biopsied and the frozen tissue sections were cut with a cryostat to a thickness of 8 μ m. The fluorescence microscopy was carried out using an Axioplan microscope (Zeiss, Germany) with a 100 W mercury lamp. The fluorescence images were recorded by a light-sensitive thermo-electrically cooled charge coupled device (CCD) camera (resolution: 385x578 pixels with a dynamic range of 16 bits per pixel) (Astromed CCD 3200, Cambridge, UK) and hard copies on a video printer (Sony multiscan video printer UP-930). The filter combination used for detection of porphyrin fluorescence consisted of 390-440

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nm excitation filter, a 460 nm beam splitter and a >600 nm emission filter.

Results

PpIX fluorescence was measured in situ by an optical-fiber based system in the normal skin of nude mice 0.5, 1, 1.5, 2.5, 3, 3.5 and 14 hours after topical application of free ALA or one of its ester derivatives as described above. As shown in Figure 1, the PpIX fluorescence was already built-up 1 hour after topical application in the case of all derivatives, while the fluorescence was seen 1.5 hours after the application of free ALA. The maximum fluorescence intensity was found 14 hours after the application in all cases, but PpIX fluorescence induced from ALA esters in the skin was stronger than that from free ALA. Furthermore, as can be seen in Figure 2, 14 hours after the application no fluorescence of ALA-esters-induced PpIX was detected in other areas of the skin and internal organs including ear, dermis, muscle, brain and liver. However, in the case of free ALA, a strong fluorescence was also seen in the ear as well as in the other areas of the skin. Thus, after topical application ALA-ester-induced PpIX was found locally in the skin, whereas free ALA-induced PpIX distributed not only locally, but also in other areas of the skin. We suggest that ALA is transported in the blood and that PpIX is subsequently formed in all organs containing the enzymes of the heme synthesis pathway and/or PpIX is formed in the skin and then transported to other tissues via blood circulation. The latter situation may lead to skin photosensitivity in areas where free ALA is not topically applied. In addition, after intraperitoneal injection of ALA methylester at a dose of 150 mg/kg, the PpIX fluorescence in the skin of mice was built-up 15 minutes after the injection and the peak value was found around

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4 hours, and the fluorescence disappeared within 10 hours post the injection (Figure 3). This kinetic pattern is similar to that of the fluorescence of free ALA-induced porphyrins in the skin following i.p. injection of the same dose, although the fluorescence decreased faster in the case of the ester than in the case of the free ALA.

Example 10

Measurements of protoporphyrin IX production in human basal cell carcinoma (BCC) and surrounding normal skin by optical-fiber based system

The PpIX fluorescence in the BCC lesions and surrounding normal skin of human patients was measured in situ by optical-fiber based system after topical application of 20% free ALA and its derivatives for various time intervals.

Figures 4, 5, 6 and 7 show that, compared to free ALA, the ALA derivatives-induced PpIX was built up faster, produced more and localized more selectively in the BCC lesions (i.e. much less fluorescence in the surrounding normal skin), particularly for ALA methylester.

Example 11

In vivo fluorescence surface measurements of PpIX production in human BCC and surrounding normal skin by CCD microscopy of biopsies

In a 78 years old Caucasian male presenting multiple ulcero-nodular BCCs lesions were exposed to commercial oil-in-water creams containing either ALA alone (20% w/w) or ALA methyl ester (20% w/w) (as described in Example 7) covered by a semi-permeable dressing for 24 hours. After removal of dressings and cream in vivo fluorescence was measured at the surface of tumor tissue

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and adjacent normal skin by means of a spectrofluorometer. Punch biopsies of the same areas were removed and samples were immediately immersed in liquid nitrogen. The tissue sections were cut with a cryostat microtome to a thickness of 8 μm . The localization pattern of the porphyrin fluorescence in the tissue sections was directly observed by means of fluorescence microscopy. The same frozen sections were subsequently stained with routine H&E staining for histological identification. The same sections were subsequently stained with routine H&E staining for histological identification. Fluorescence microscopy was carried out with an Axioplan microscope (Zeiss, Germany). Fluorescence images and quantitative measurements were performed by a light-sensitive thermol-electrically cooled charge coupled device (CCD) camera (Astromed CCD 3200, Cambridge, UK) and an image processing unit (Astromed/Visilog, PC 486DX2 66 MHz VL). The main purpose for such quantitative measurements is to determine the exact penetration of ALA-induced porphyrins from tissue surface to the bottom layers of cancer lesions. The results are shown in Figures 8 and 9 in which the fluorescence intensity is expressed as a function of depth of cancer lesion.

As shown in Figures 8 and 9, an homogeneous distribution of PpIX fluorescence is seen from the top to the bottom of the whole BCC lesions after use of either free ALA or its methyl ester. This suggests that ALA methylester is at least as good as free ALA in terms of penetration and PpIX production in the BCC lesion. In addition, no PpIX fluorescence was seen in the surrounding normal skin after topical application of ALA methylester, indicating that ALA-methylester-induced PpIX highly selectively took place only in the BCC lesion.

In vivo fluorescence after 24 hours showed at least

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doubled fluorescence intensity for ALA methyl ester compared to ALA for the selected tumors and also an increase for corresponding normal tissues, however this only of about 50%. The ratio between tumor and normal tissue was about 1.2: 1 for ALA and 2: 1 for the ALA methyl ester. The results are shown in Figures 10 and 11.

At control one week after treatment all treatment fields presented a central necrotic area corresponding to the tumor. In the adjacent normal skin exposed to cream and light irradiation there was observed a marked erythema for the ALA while for the ALA methyl ester only moderate erythema was observed.

Example 12

In vivo fluorescence surface measurements of PpIX production in human BCC and surrounding normal skin by CCD microscopy of biopsies

The present data show the localization patterns and production of porphyrins (mainly protoporphyrin IX (PpIX)) after topical application of free ALA and one of its derivatives (methyl ester) for 4.5 and 24 hours in the nodular basal cell carcinomas (BCCs) and surrounding normal skin of patients. The tests were performed as described in Example 11.

Each of the following figures show both (A) fluorescence images of either the bottom layer of BCC lesions or of the surrounding normal skin. Curves indicating the fluorescence intensity as a function of depth of the BCC lesions or of the normal skin are also shown (B).

Figure 12 shows a homogenous distribution of PpIX fluorescence generated by ALA methyl ester in the bottom

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layer of a BCC 4.5 hours after topical application. There is also some porphyrin fluorescence in surrounding normal skin (Figure 13). The fluorescence intensity ratio between BCC and the normal skin is about 2. Moreover, the absolute amount of the fluorescence induced by ALA methyl ester is higher than that induced by free ALA and 20% DMSO after topical application for 4.5 hours, as shown below.

Figures 14 and 15 show a uniform distribution of porphyrin fluorescence induced by topical application of ALA methyl ester for 24 hours in the bottom layer of BCC and surrounding normal skin. The ratio of the fluorescence in BCC and that in normal skin is also about 2. Furthermore, the fluorescence intensity of ALA methyl ester-induced porphyrins in the BCC is almost twice as high as that in BCC after topical application of free ALA alone for 24 hours, as shown below.

Figures 16 and 17 show a homogenous distribution of free ALA-induced porphyrins in the bottom layer of BCC and surrounding normal skin 24 following topical application. However, the ratio of the fluorescence intensity between BCC and normal skin is about 1, which indicates a low selectivity of this treatment. Moreover the production of porphyrins in BCC is less than that in the case of ALA methyl ester.

Figures 18 and 19 show a homogenous distribution of ALA-induced porphyrins in the bottom layer of BCC and surrounding normal skin after topical application of free ALA and 20% DMSO for 4.5 hours. However, the ratio of the fluorescence intensity between BCC and normal skin is only slightly larger than 1, which demonstrates that the DMSO probably reduces the tumor selectivity of the porphyrins produced. Moreover, also in this case less porphyrins are produced in BCC than in the case of

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the application of ALA methyl ester.

Example 13

Investigation of the effects of the chelating agent desferrioxamine (DF) and/or DMSO and fluorescence of skin

I. The effect of DF and/or DMSO on the build up of fluorescence in the normal skin of mice in situ was ascertained various times after topical administration of ALA-methylester. Methods were performed as described in Example 9.

RESULTS

Topical application of the cream alone containing only DMSO did not show any fluorescence in the normal mouse skin, but there was some fluorescence of PpIX after DF alone was applied.

DF or DF plus DMSO (a well-known skin penetration enhancer) significantly enhanced the production of ALA methylester-induced PpIX.

II. Fluorescence imaging of the skin treated with three derivatives (performed as described in Example 9) showed fluorescence of the ester derivative-induced porphyrins in the epidermis, epithelial hair follicles and sebaceous glands 1 h after topical application (Figure 21). The fluorescence intensity of the porphyrins increased with the time after the application.

SUMMARY

A large number of patients with basal cell carcinomas (BCCs) has topically been treated with ALA-based PDT in our hospital during the past five years and more than 90% of superficial BCCs have shown a complete

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regression. However, nodular BCCs had a low complete response rate due to a poor ALA retention and, consequently, a low ALA-induced porphyrin production in the deep layers of the lesions. In order to improve the technique, we used ALA ester derivatives instead of free ALA. The present data obtained presented in this Example and in Example 9 by means of both fluorescence spectroscopic measurements in situ and fluorescence microscopy of tissue biopsies, indicate that all three ester derivatives studied were taken up, de-esterified and finally converted into porphyrins in the epidermis, epithelial hair follicles and sebaceous glands of the nude mice with a higher porphyrin production than that of free ALA. This is in agreement with the preceding Examples concerning a study of human nodular basal cell carcinoma that demonstrate that the fluorescence of the ALA ester-induced porphyrins was built up faster with a higher intensity and a more homogenous distribution than those of free ALA-induced porphyrins in the lesions.

The present study also shows that DF has a significant effect in enhancing the production of ALA methylester-derived PpIX in the normal skin of the mice after topical application.

Interestingly, a strong fluorescence of free ALA-induced porphyrins was found in regions of the skin outside the area where the cream was topically applied (Figure 2). This indicates that after topical application free ALA is transported in the blood and porphyrins are subsequently formed in all organs containing the enzymes of the heme synthesis pathway or porphyrins are initially formed in the skin or/and liver, then transported to other tissues via blood circulation. This may lead to skin photosensitivity in areas where free ALA is even not topically applied. However, none of the ester derivatives studied induced porphyrin

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fluorescence in other parts of the skin.

Example 14

Effects of ALA methylester or ALA, DF and DMSO PDT on tumor growth in WiDr human colonic carcinoma-transplanted nude mice

Nude mice were transplanted with WiDr human colonic carcinoma cells by subcutaneous injection into the right flank region. The following creams, formulated as described in the preceding Examples, were applied topically to the site of the tumor: 10% DF alone; 20% ALA + 10% DF + 20% DMSO; or 20% ALA methylester + 10% DF + 20% DMSO, followed, 14 hours later by laser light irradiation (632 nm, 150 mW/cm² for 15 minutes). A separate group of animals bearing the same tumor model, but receiving no topical application of the cream, served as a control. The responses of the treated tumors were evaluated as tumor regression/regrowth time. When the tumors reached a volume 5 times that of the volume on the day of light irradiation, the mice were killed. The results are shown in Figure 22. (Bars: standard error of mean (SEM) based on 3-5 individual animals in each group). The results show that it took 34 days for tumors treated with ALA methylester + DF + DMSO to reach a volume five times that of the volume on the day just before light irradiation, whereas in the case of free ALA + DF + DMSO it took 24 days for the treated tumors to grow to 5 times size. Thus, ALA methylester is more effective than ALA in slowing tumor regrowth.

EXAMPLE 15

Selectivity of ALA esters (methyl, hexyl, heptyl and octyl) for non-normal tissue

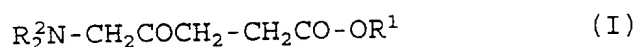
1. **Abstract** (100 words) – Summarize the main findings of the study.
 2. **Introduction** (200 words) – Provide background information and state the research objectives.
 3. **Methods** (150 words) – Describe the study design, participants, and procedures.
 4. **Results** (250 words) – Present the data and statistical analyses.
 5. **Discussion** (200 words) – Interpret the findings and discuss their implications.
 6. **Conclusion** (50 words) – Summarize the key takeaways.
 7. **References** (100 words) – List the sources cited in the paper.

1. *Abstracts* 1-2
 2. *Abstracts* 3-4
 3. *Abstracts* 5-6
 4. *Abstracts* 7-8
 5. *Abstracts* 9-10
 6. *Abstracts* 11-12
 7. *Abstracts* 13-14
 8. *Abstracts* 15-16
 9. *Abstracts* 17-18
 10. *Abstracts* 19-20
 11. *Abstracts* 21-22
 12. *Abstracts* 23-24
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 14. *Abstracts* 27-28
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 98. *Abstracts* 195-196
 99. *Abstracts* 197-198
 100. *Abstracts* 199-200

Claims:

1. Compounds being esters of 5-aminolevulinic acids or pharmaceutically acceptable salts thereof for use in photochemotherapy or diagnosis.

2. Compounds of formula I,



(wherein R¹ may represent alkyl optionally substituted by hydroxy, alkoxy, acyloxy, alkoxycarbonyloxy, amino, aryl, oxo or fluoro groups and optionally interrupted by oxygen, nitrogen, sulphur or phosphorus atoms; and R², each of which may be the same or different, represents a hydrogen atom or a group R¹) and salts thereof for use in photochemotherapy or diagnosis.

3. Compounds as claimed in claim 2 wherein the aryl group is phenyl or a monocyclic 5-7 membered heteroaromatic.

4. Compounds as claimed in claim 2 or 3 wherein R¹ represents an unsubstituted alkyl group and/or each R² represents a hydrogen atom.

5. Compounds as claimed in any one of claims 2 to 4 wherein the alkyl group contains up to 10 carbon atoms.

6. Compounds as claimed in any one of claims 2 to 5 wherein the compounds are ALA-methylester, ALA-ethylester, ALA-propyl ester, ALA-hexylester, ALA-heptylester or ALA-octylester or salts thereof.

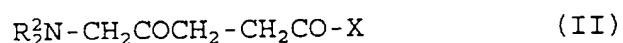
7. A process for preparing the compounds as defined in any one of claims 1 to 6, comprising forming an ester of the carboxy group of a 5-aminolevulinic acid.

AMENDED SHEET

8. A process as claimed in claim 7, comprising reacting a 5-aminolevulinic acid, or an esterifiable derivative thereof, with an alkanol or an ester-forming derivative thereof.

9. A process as claimed in claim 7 or 8, which process comprises at least one of the following steps:

(a) reacting a compound of formula II



(wherein X represents a leaving group, or COX represents an acid anhydride group and R^2 is as defined in claim 2) with a compound of formula III



(wherein R^1 is as defined in claim 2); and

(b) converting a compound of formula I into a pharmaceutically acceptable salt thereof.

10. A pharmaceutical composition comprising a compound as defined in any one of claims 1 to 6, or a pharmaceutically acceptable salt thereof, together with at least one pharmaceutical carrier or excipient.

11. The use of a compound as defined in any one of claims 1 to 6, or a pharmaceutically acceptable salt thereof, for the preparation of a therapeutic agent for use in photochemotherapy, or a diagnostic agent for use in diagnosis.

12. The use as claimed in claim 11 wherein the photochemotherapy or diagnosis is performed on disorders

or abnormalities of external or internal surfaces of the body which are responsive to photochemotherapy.

13. A method of diagnosis or photochemotherapeutic treatment of disorders or abnormalities of external or internal surfaces of the body, comprising administering to the sites of investigation or affected surfaces, a composition as defined in claim 10, and exposing said sites or surfaces to light.

14. A method as claimed in claim 13 wherein the light is in the wavelength region 500-700 nm.

15. A product comprising a compound as claimed in any one of claims 1 to 6 or a pharmaceutically acceptable salt thereof, together with at least one surface-penetration assisting agent, and optionally one or more chelating agents as a combined preparation for simultaneous, separate or sequential use in treating or diagnosing disorders or abnormalities of external or internal surfaces of the body which are responsive to photochemotherapy.

16. A product as claimed in claim 15 wherein the surface-penetration assisting agent is DMSO.

17. A method of in vitro diagnosis of abnormalities or disorders by assaying a sample of body fluid or tissue of a patient, said method comprising at least the following steps:

- i) admixing said body fluid or tissue with a compound as defined in any one of claims 1 to 6,
- ii) exposing said mixture to light,
- iii) ascertaining the level of fluorescence, and
- iv) comparing the level of fluorescence to control levels.

AMENDED SHEET

18. A kit for use in diagnosis or photochemotherapy of disorders or abnormalities of external or internal surfaces of the body comprising:

- a) a first container containing a compound as claimed in any one of claims 1 to 6 or a pharmaceutically acceptable salt thereof,
- b) a second container containing at least one surface penetration assisting agent; and optionally
- c) one or more chelating agents contained either within said first container or in a third container.

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Free Ala on mouse

Ala Ethylester

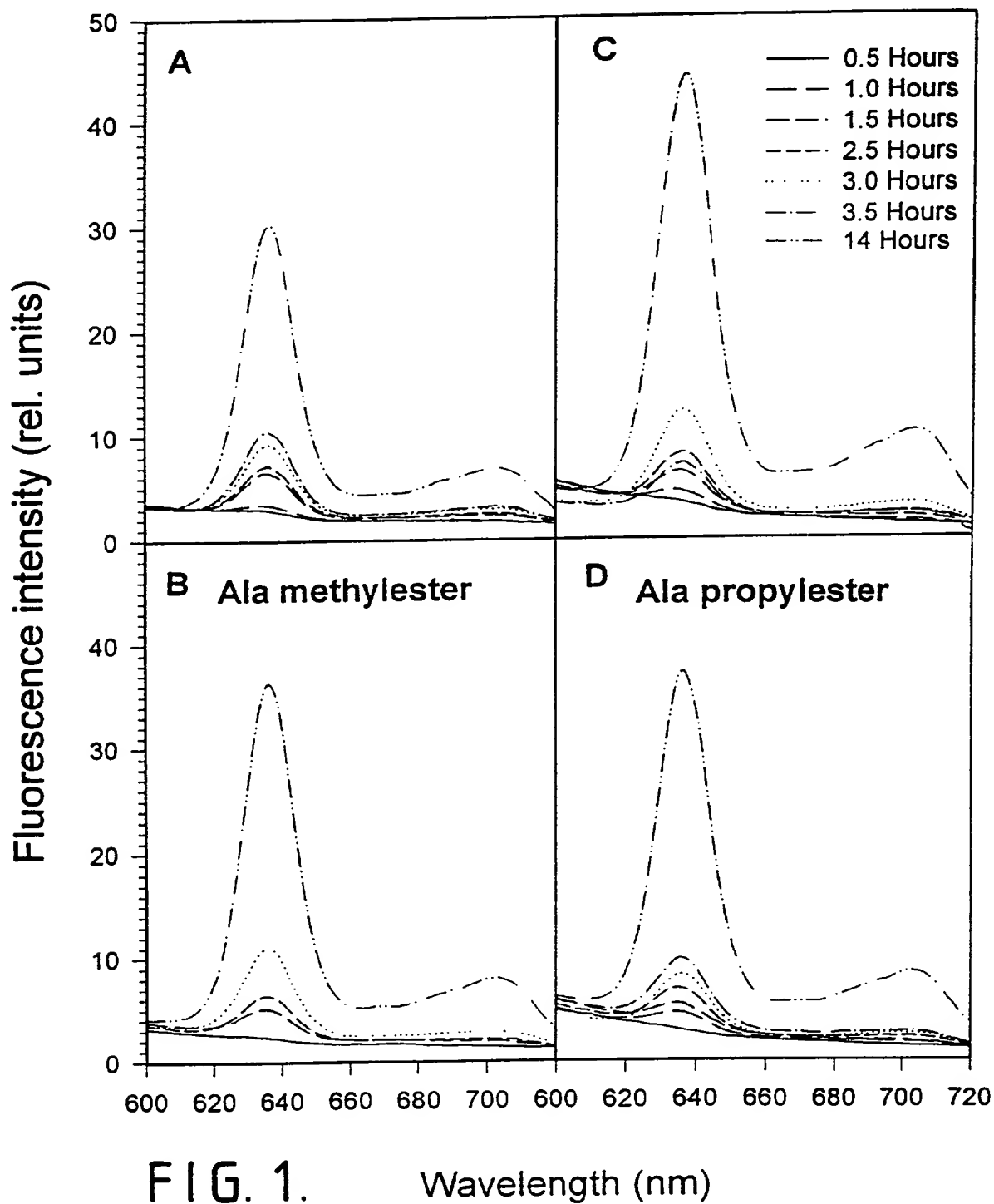


FIG. 1.

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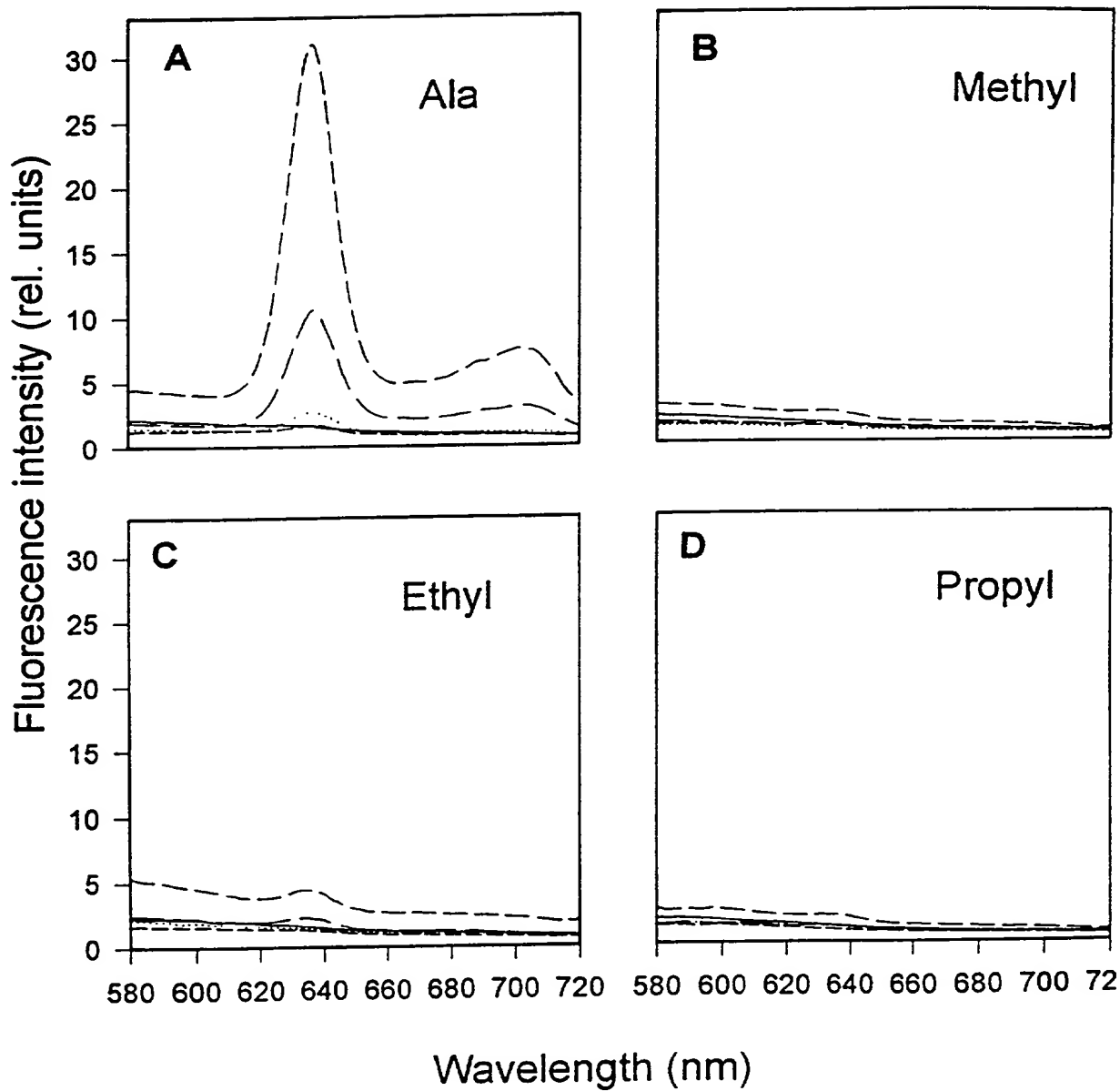


FIG. 2.

— Brain
 --- Dermis
 - - - Ear
 - . - Liver
 . . . Muscle

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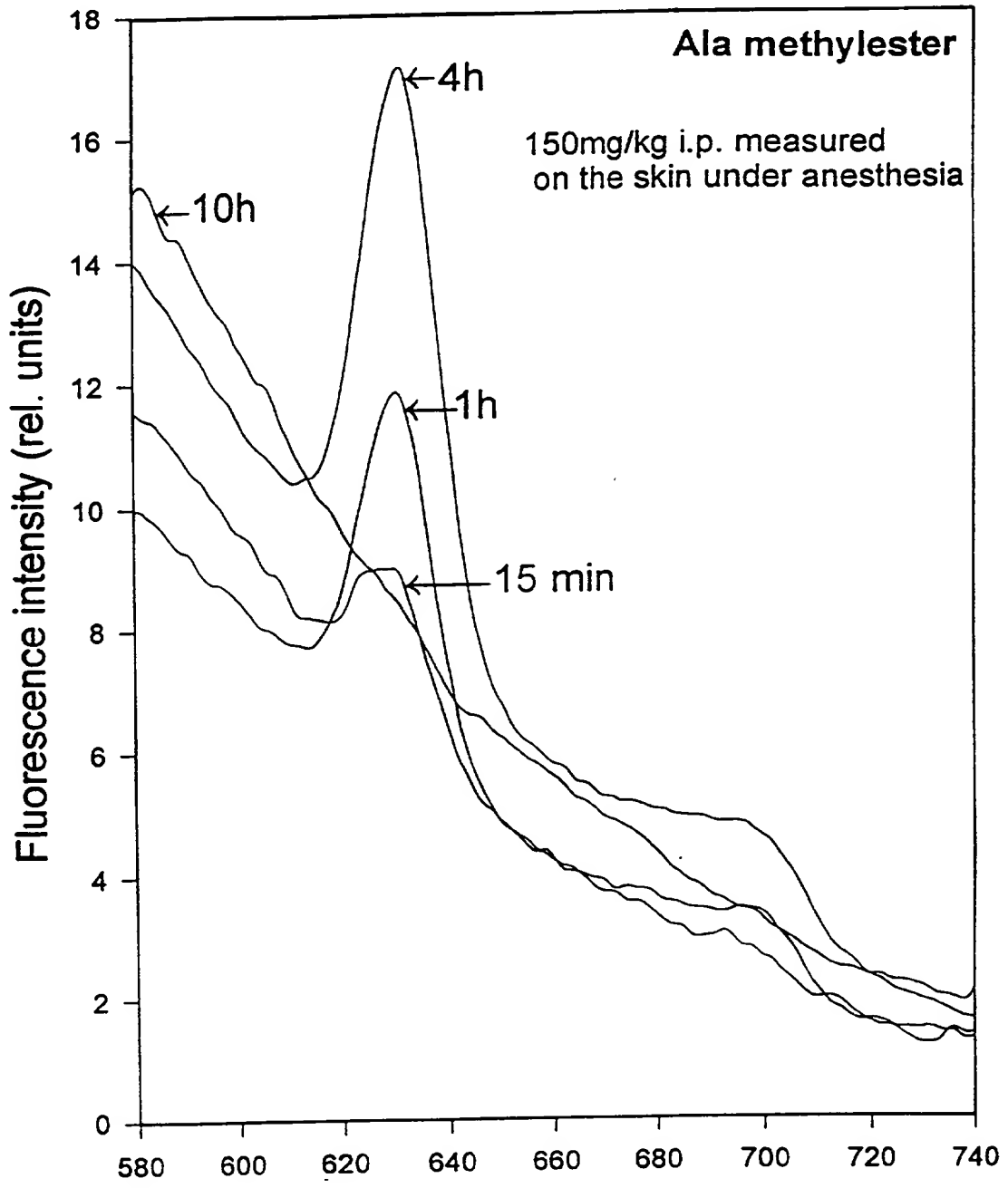


FIG. 3. Wavelength (nm)

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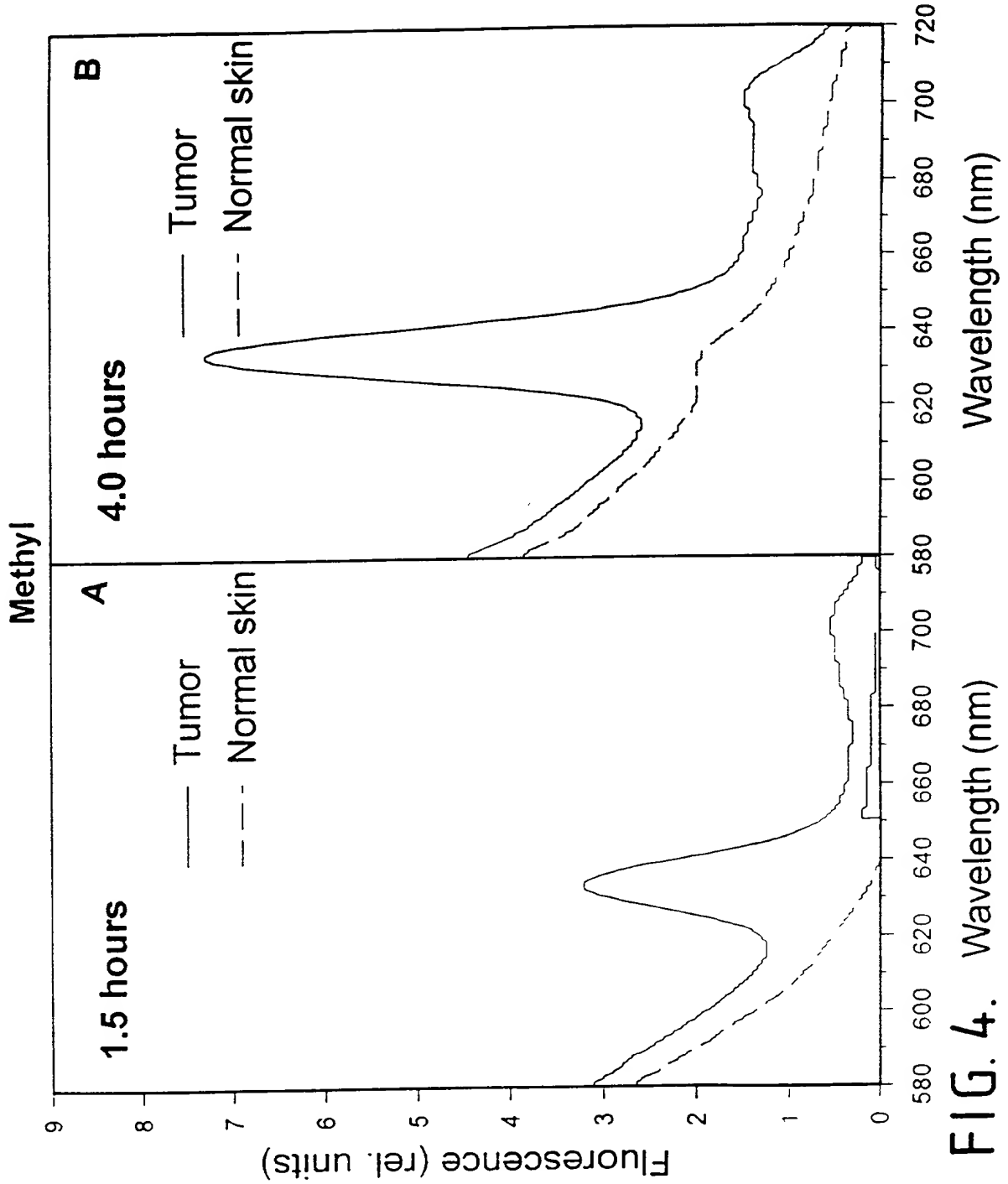


FIG. 4. Wavelength (nm)

APPROVED	O.G. FIG.
BY	CLASS. SUBCLASS
DRAFTSMAN	W09628412

PCT/GB96/00553

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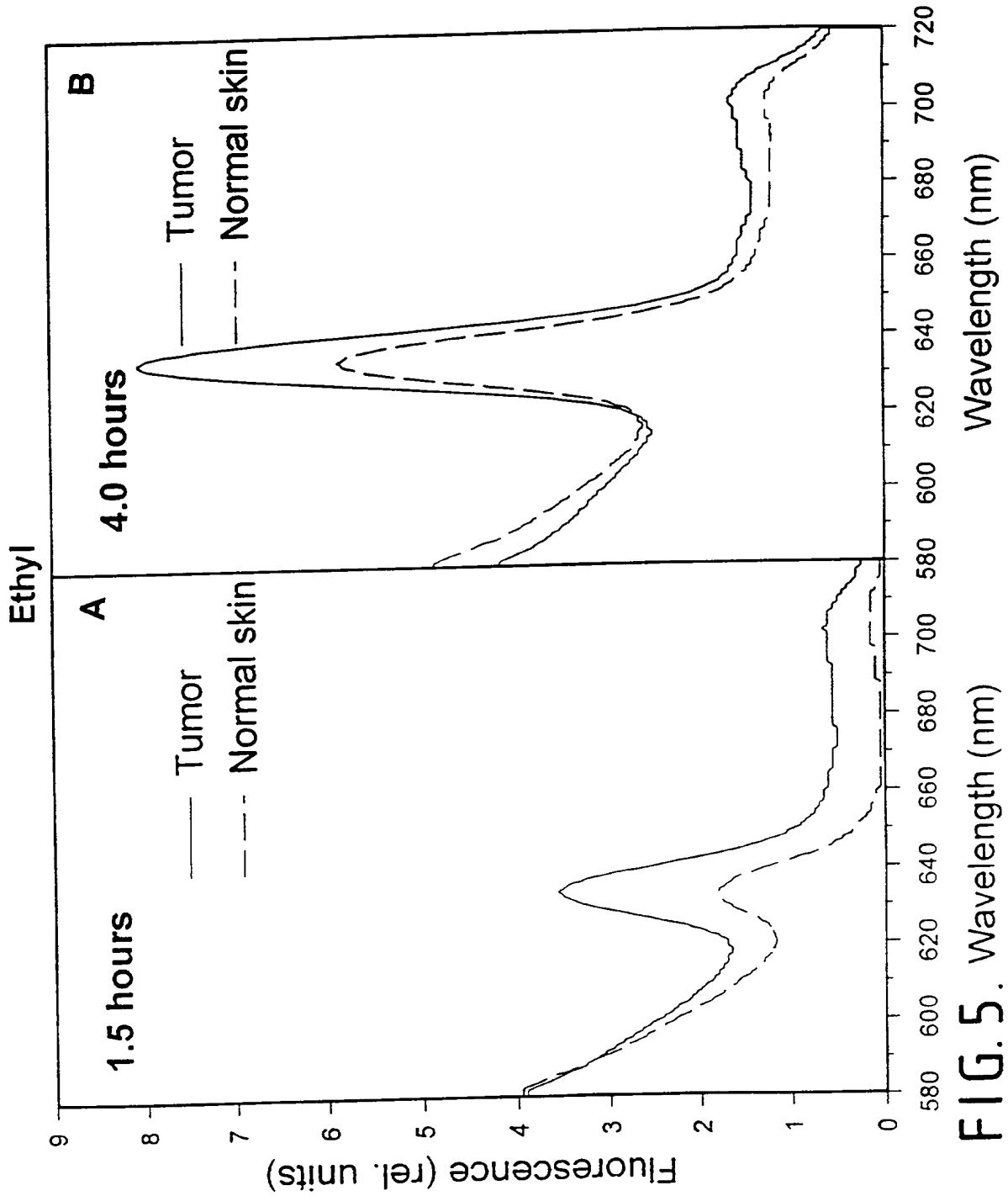


FIG. 5. Wavelength (nm)

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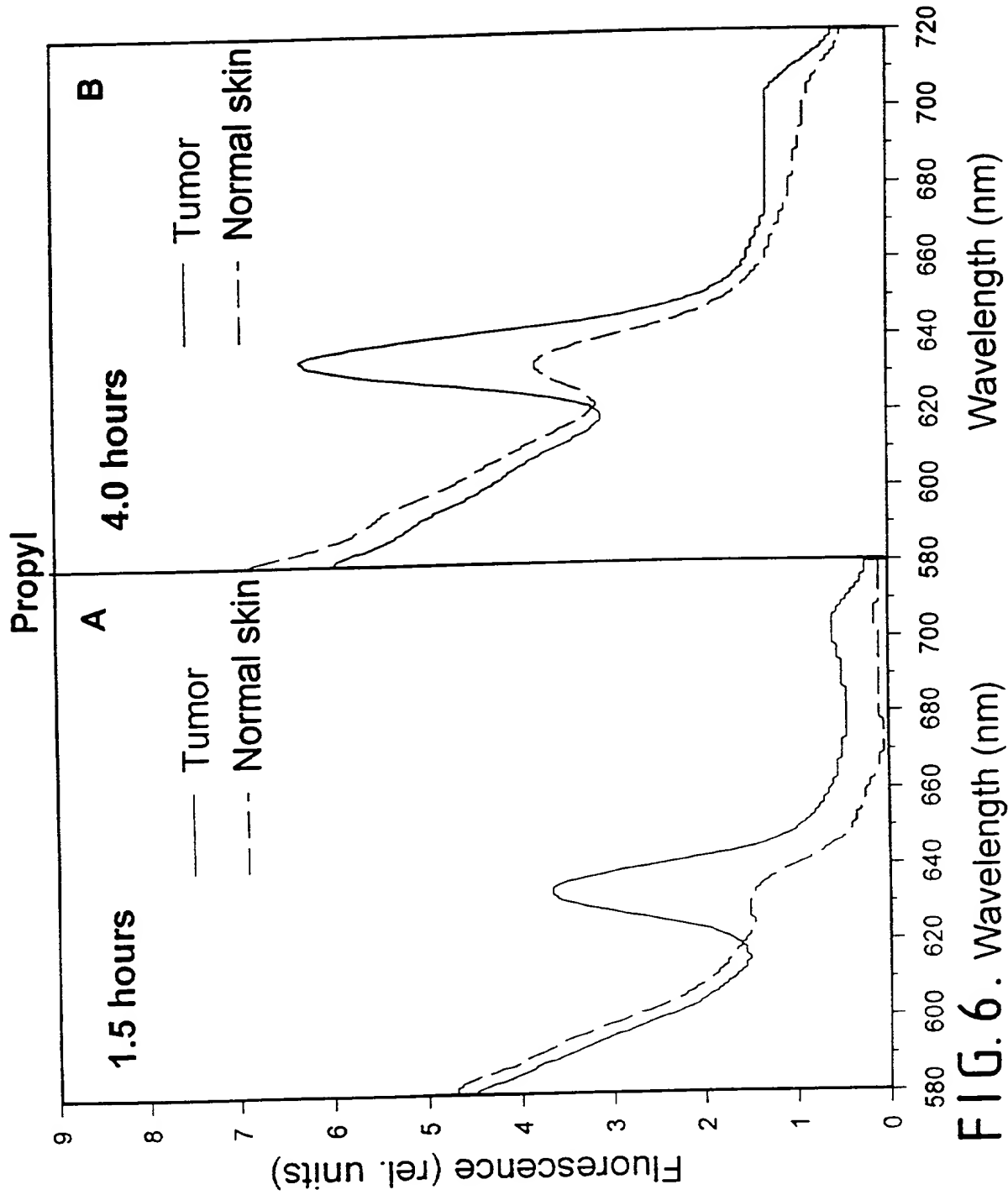
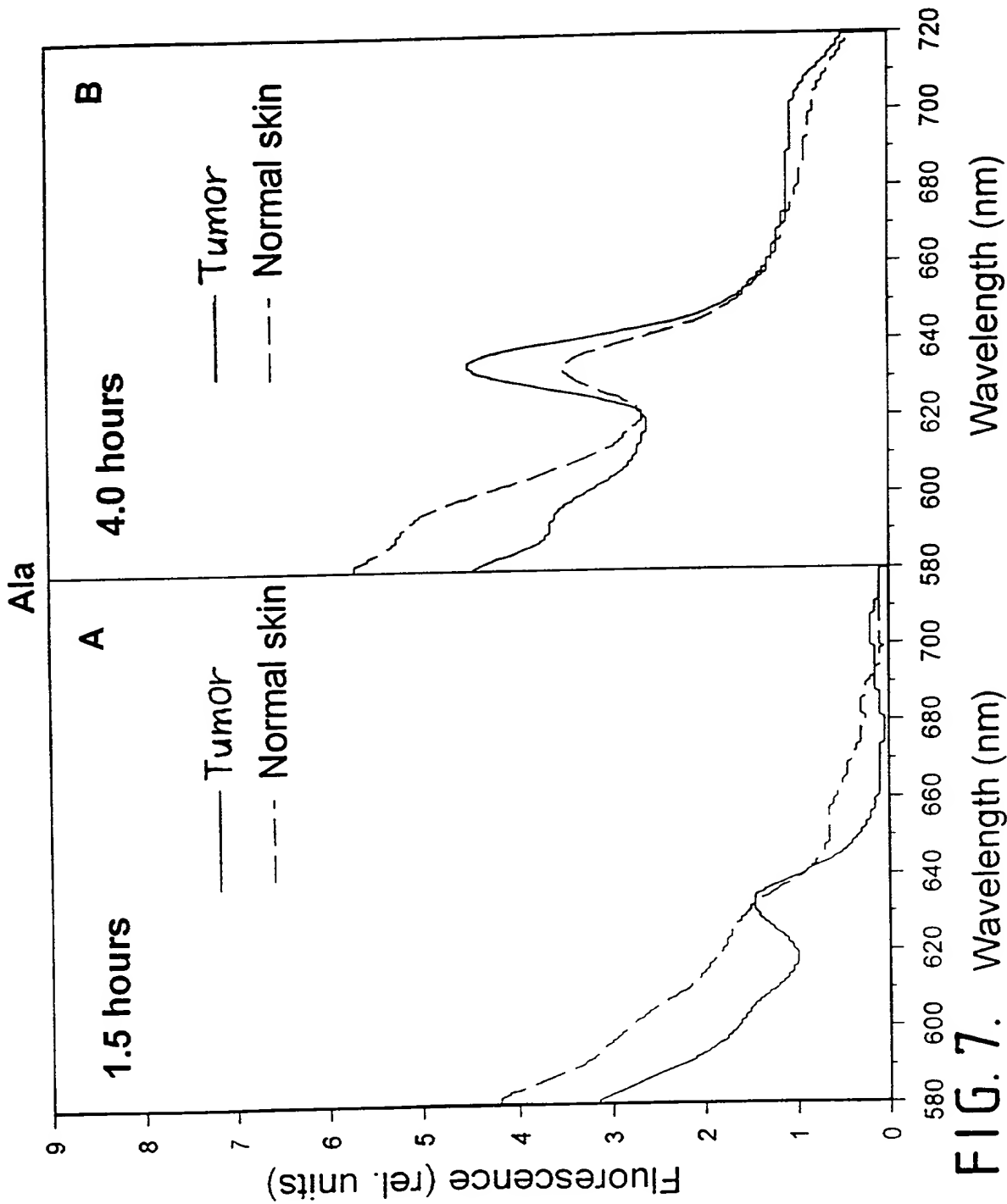


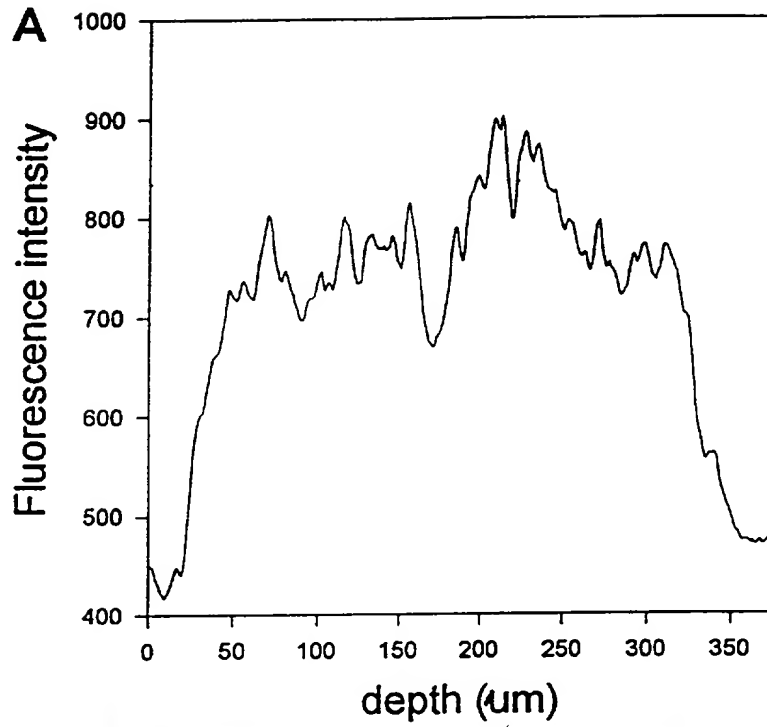
FIG. 6. Wavelength (nm)



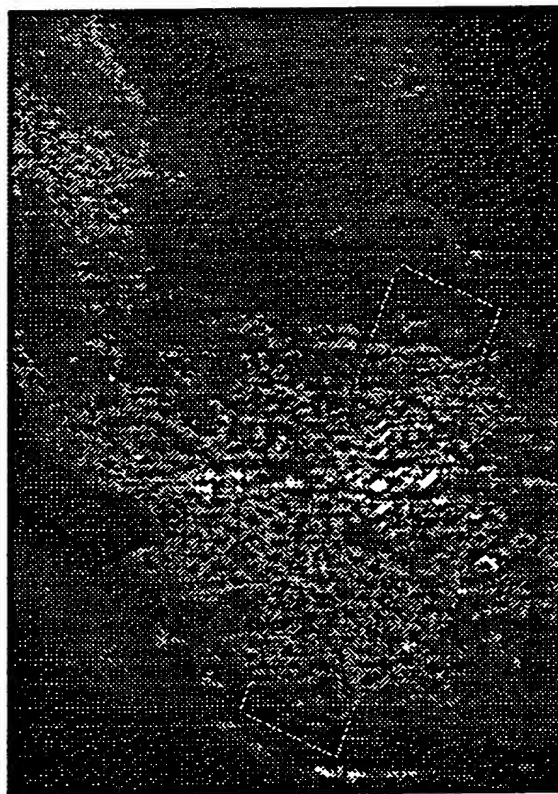
APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN	09678412	

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PCT/GB96/00553

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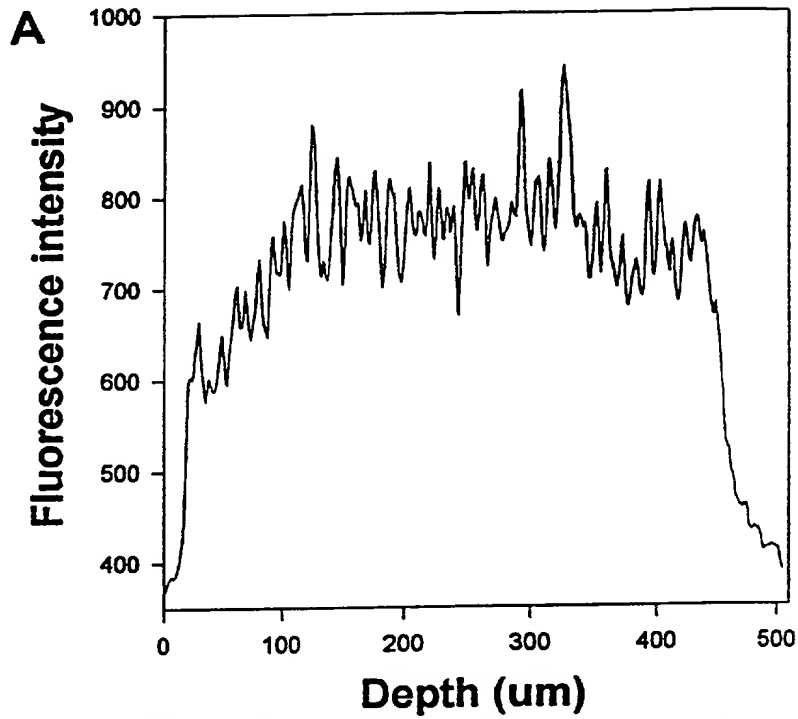
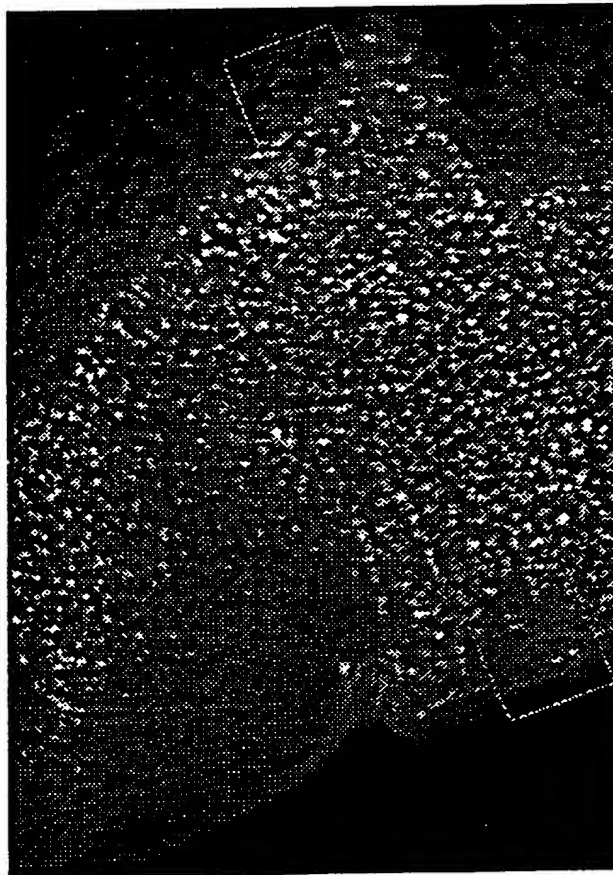
Free ALA

Figure 8

08/913257

APPROVED	O.G. FIG.
BY	CD 661812
DRAFTSMAN	CLASS

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**B****ALA methylester****Figure 9**

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BCC ALA-methyl 24 hours

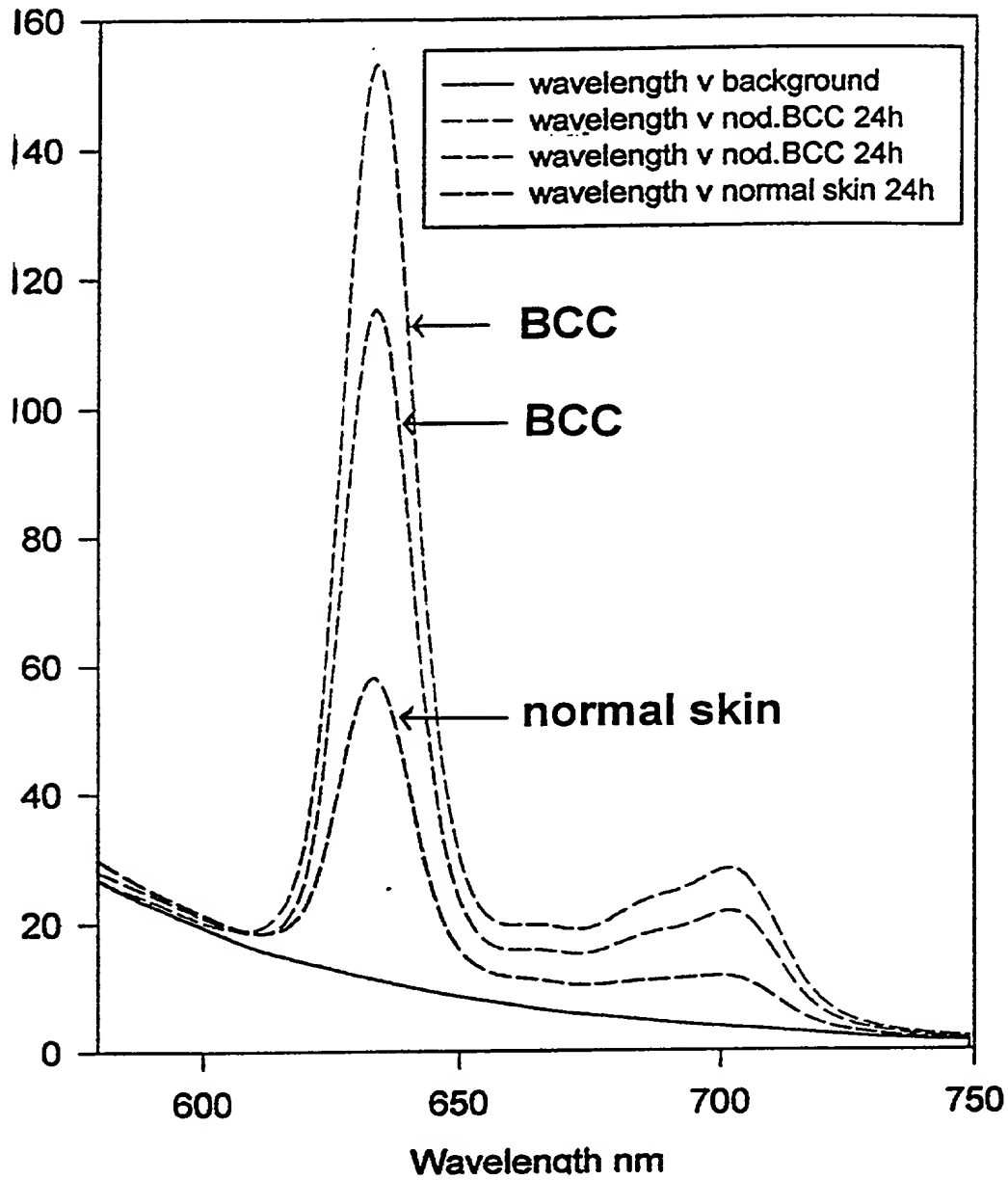


Figure 10

BCC ALA 24 hours

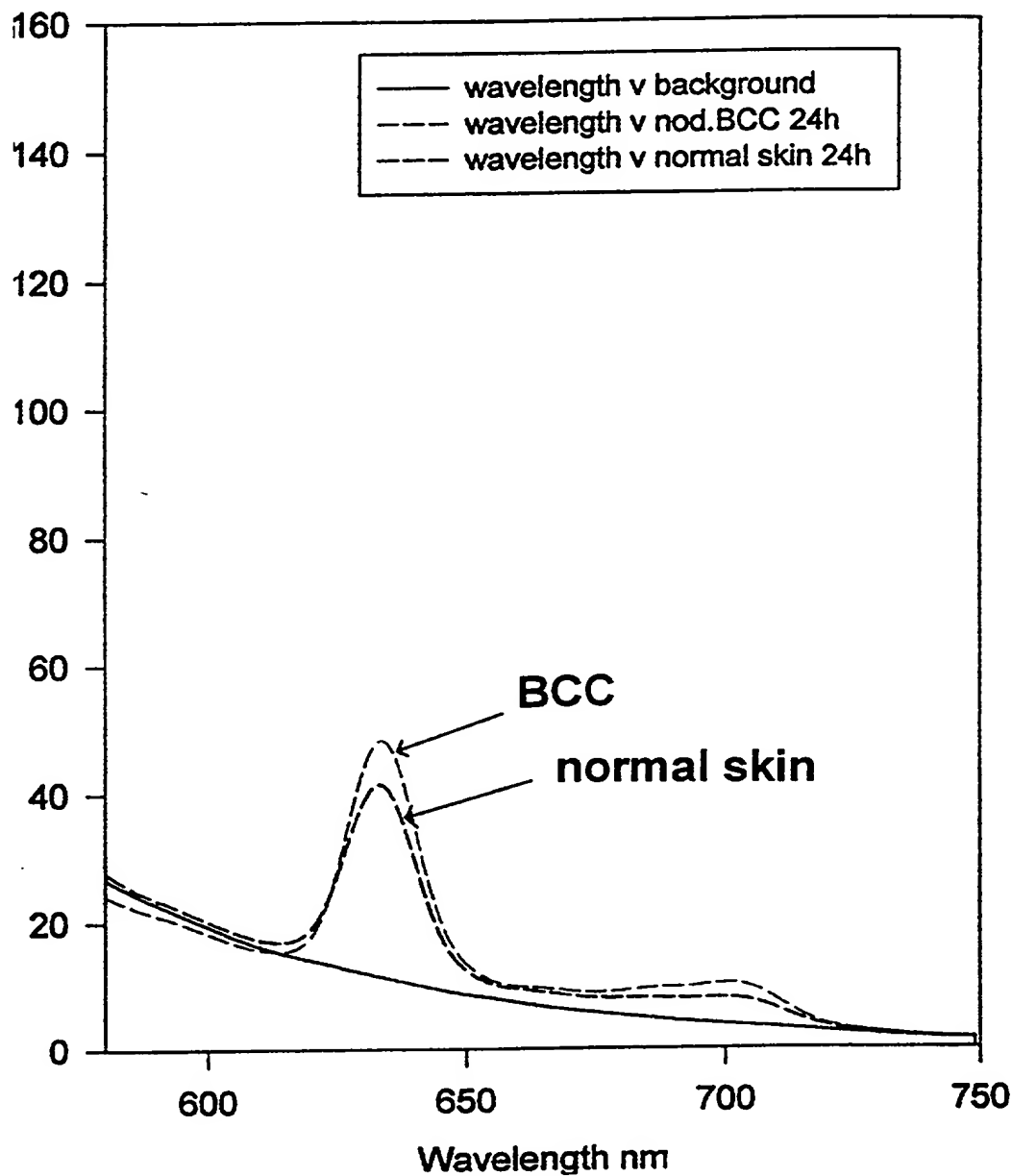


Figure 11

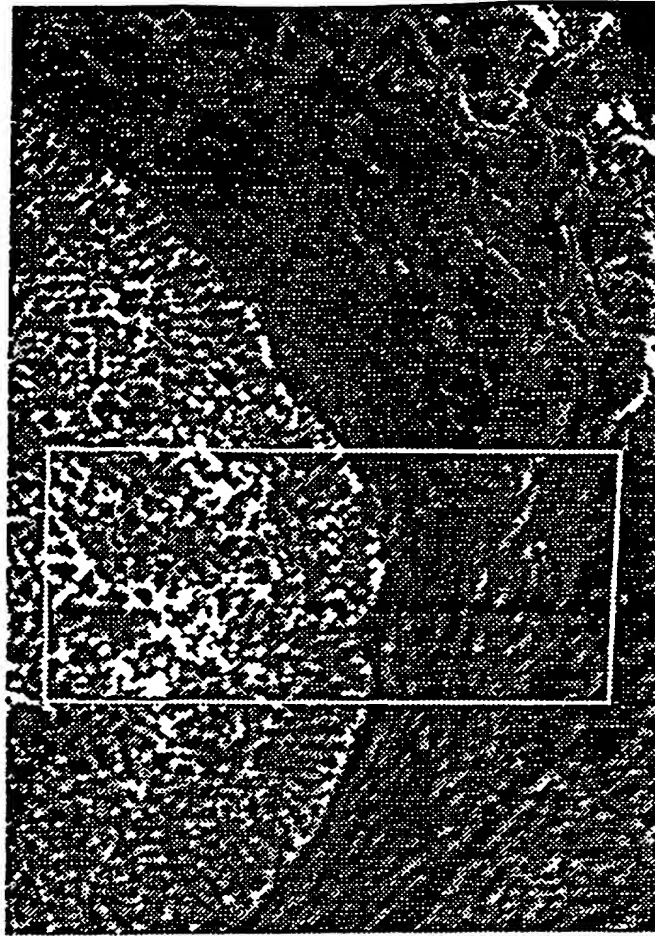
APPROVED	O.G. FIG.	
BY	1096/2849	
DRAFTSMAN	CLASS	

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PCT/GB96/00553

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B



155-20

A

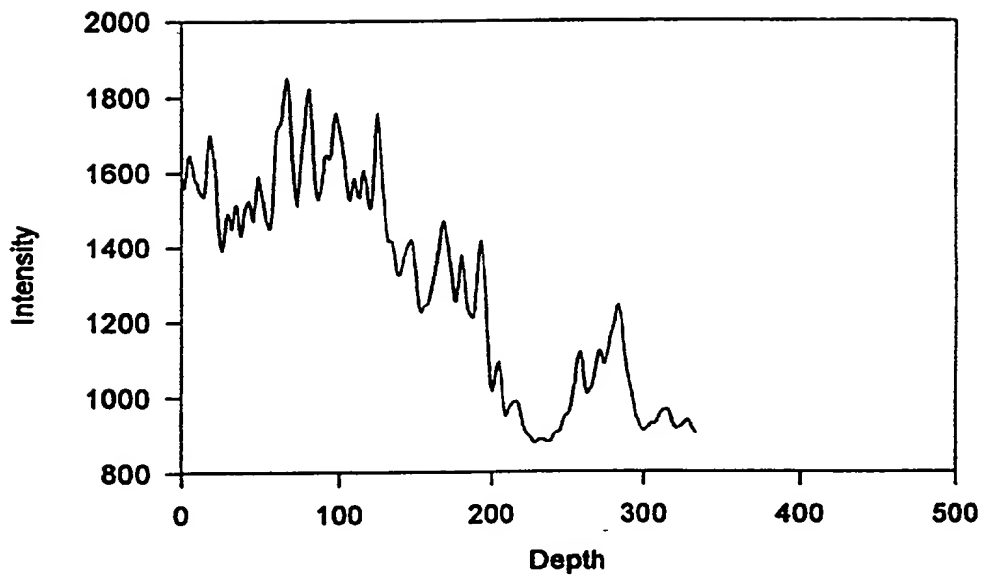
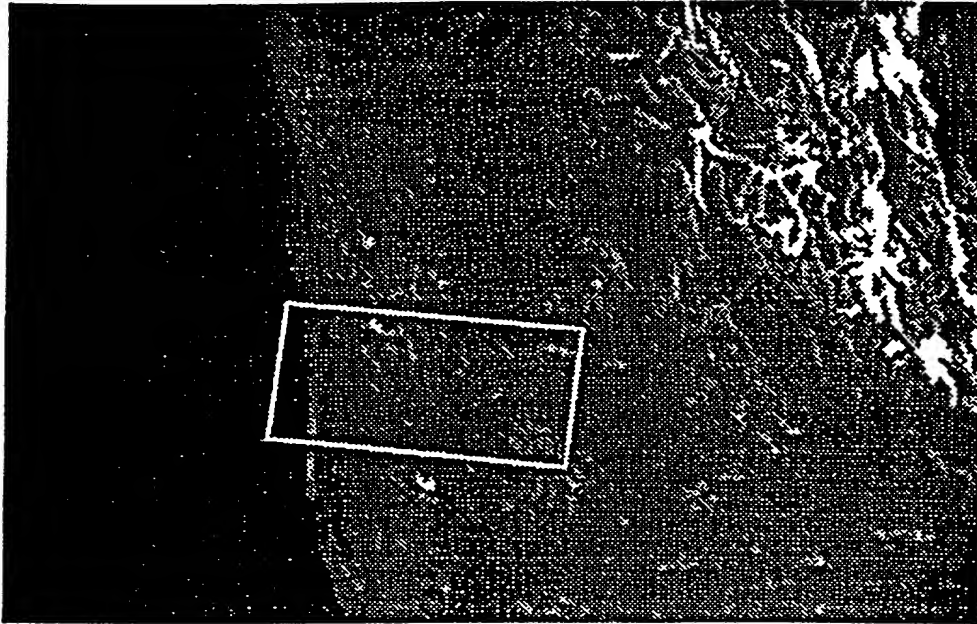


Figure 12

08/913257 1096/2849

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B



156-1

A

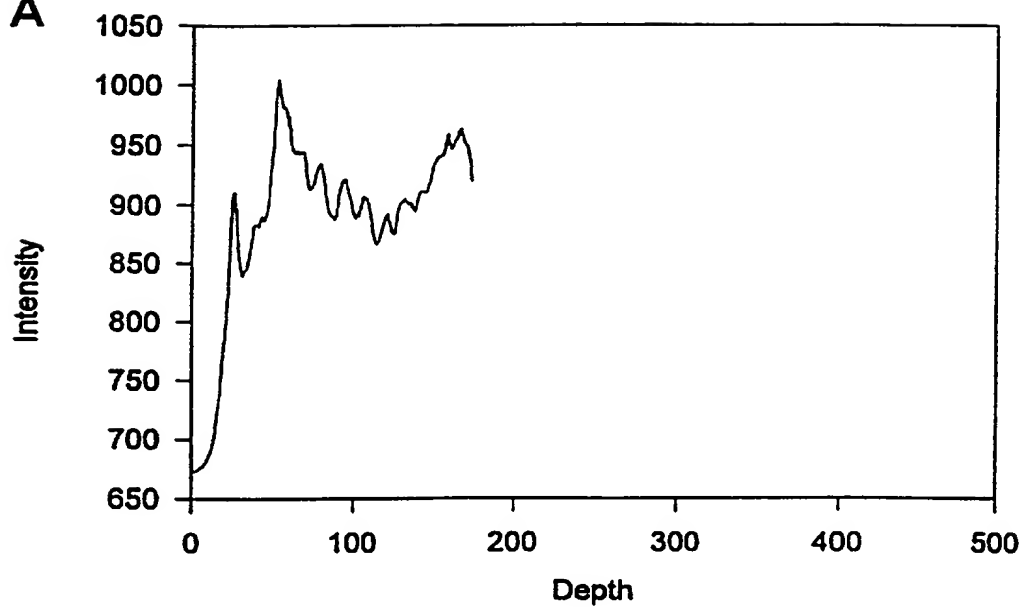


Figure 13

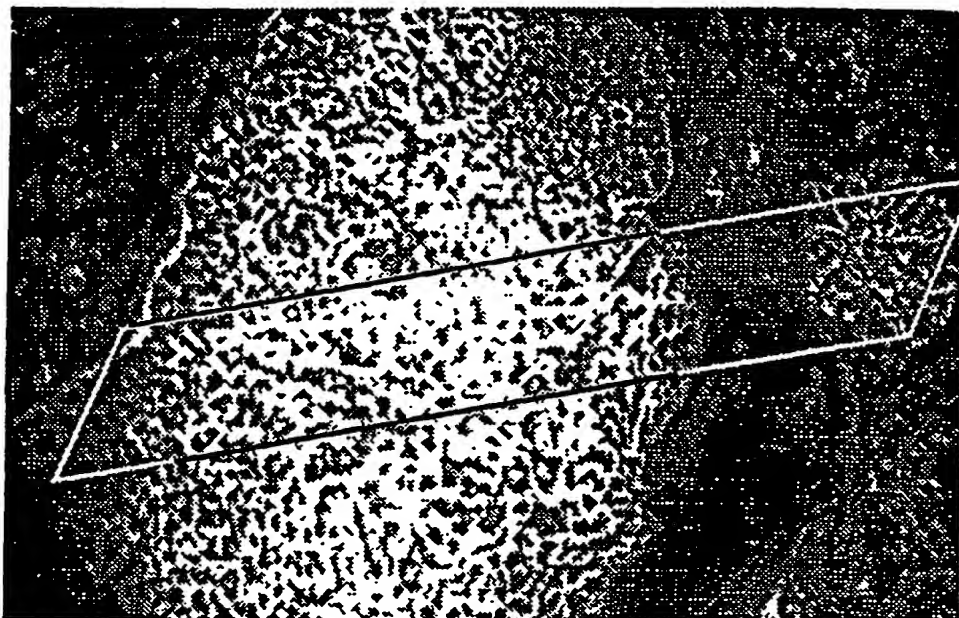
APPROVED	O.G. FIG.	
BY	0696/84	CLASS
DRAFTSMAN		

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PCT/GB96/00553

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153-11

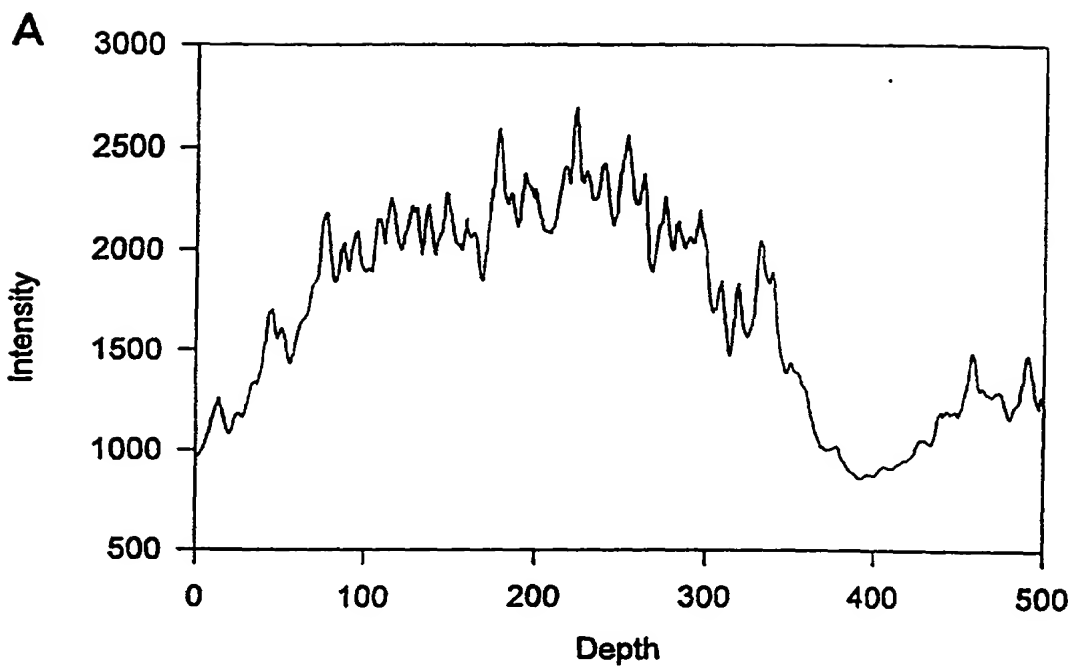


Figure 14

265021 / 5251680

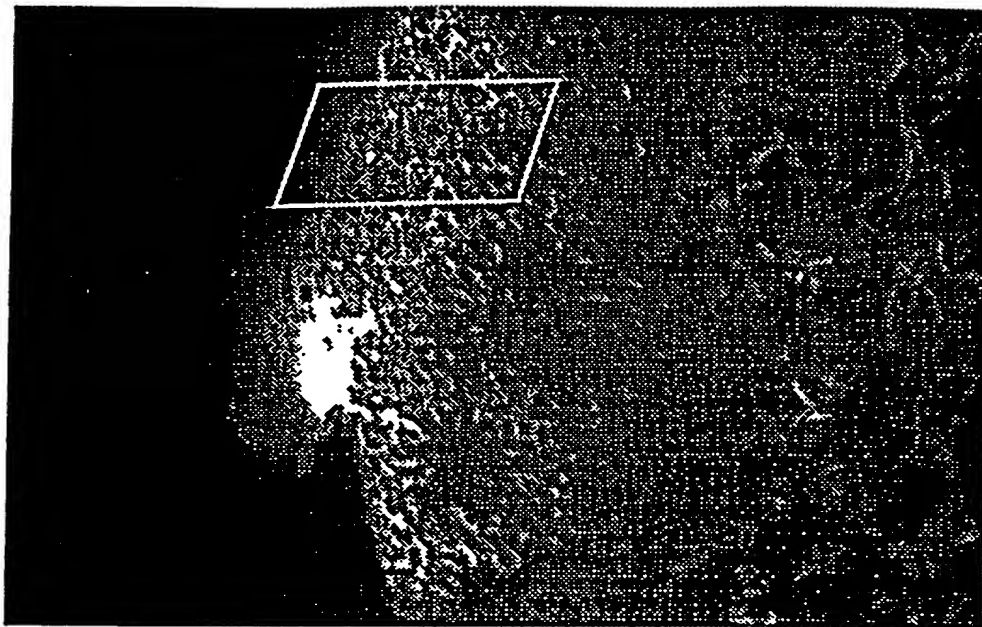
APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN	WO 96/28412	

08/913257

PCT/GB96/00553

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B



158-1

A

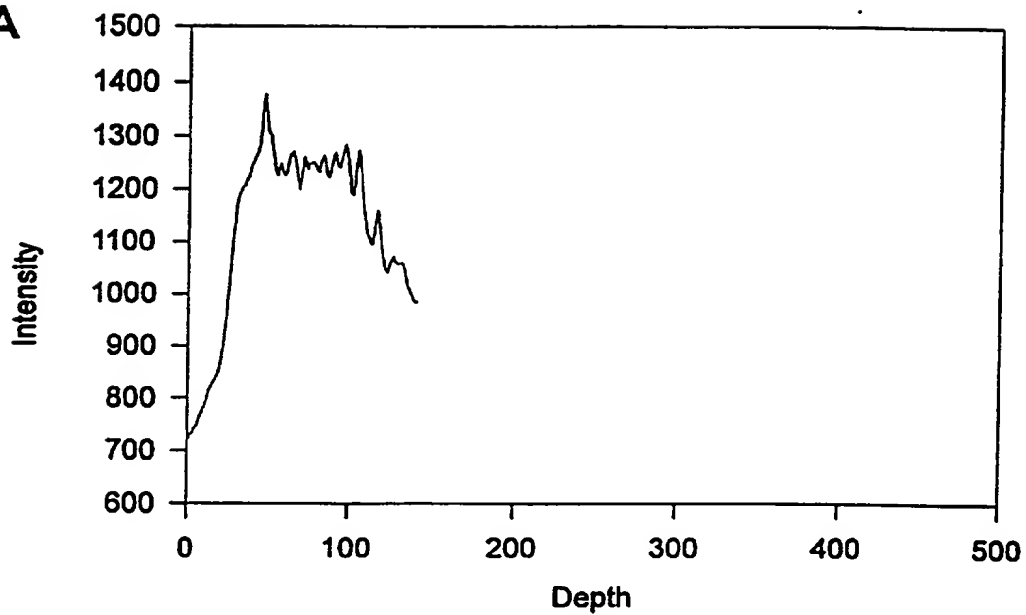
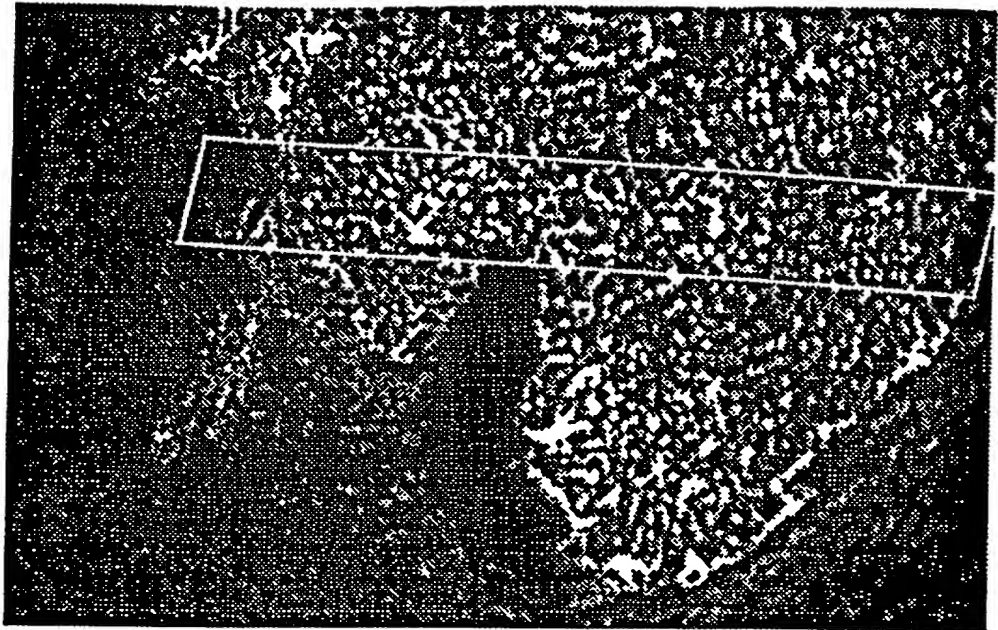


Figure 15

B



159-4

A

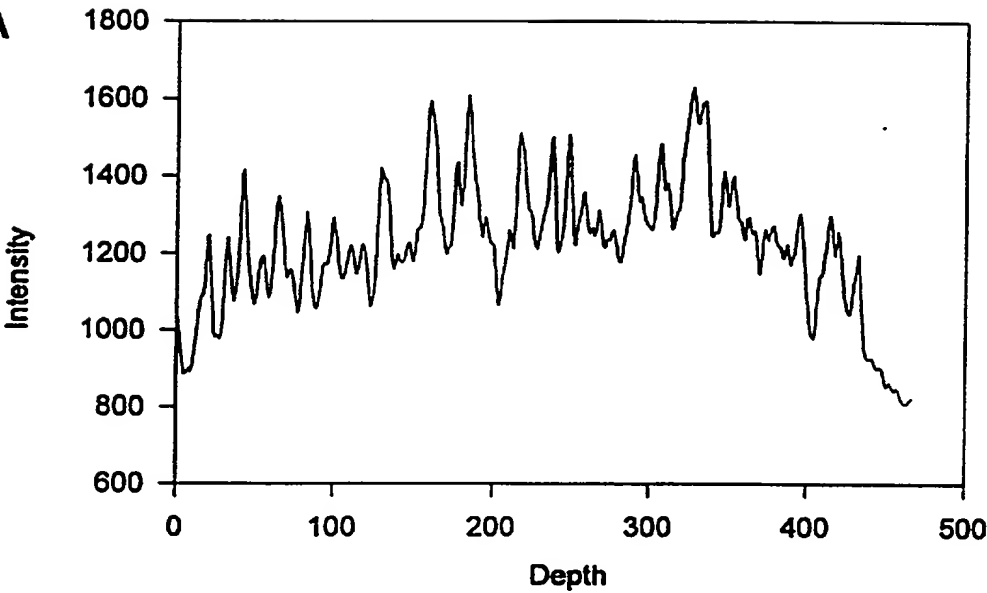
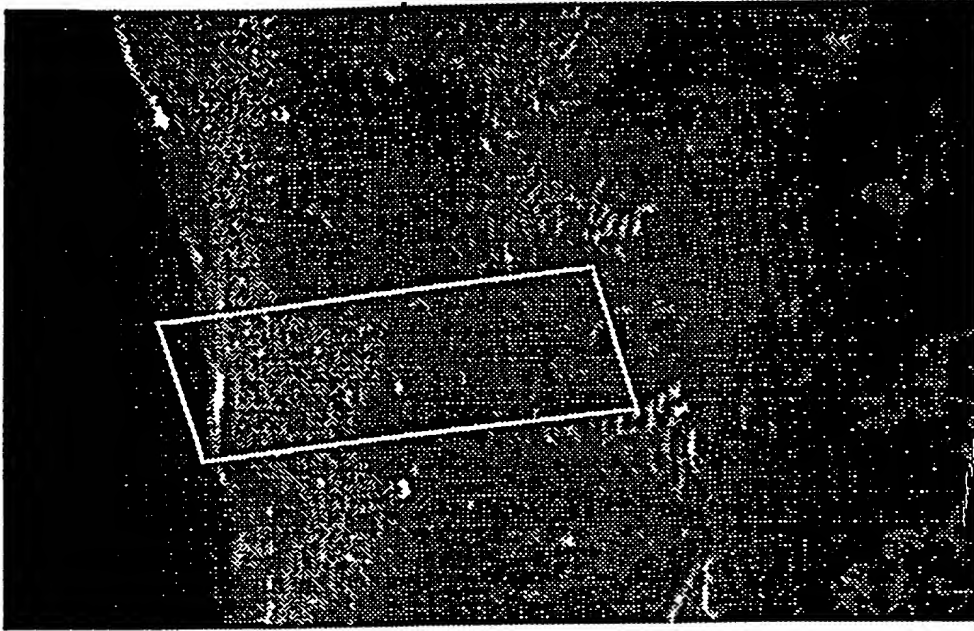


Figure 16

APPROVED	O.G. FIG.	
BY	CLASS	
DRAFTSMAN		

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B



160-1

A

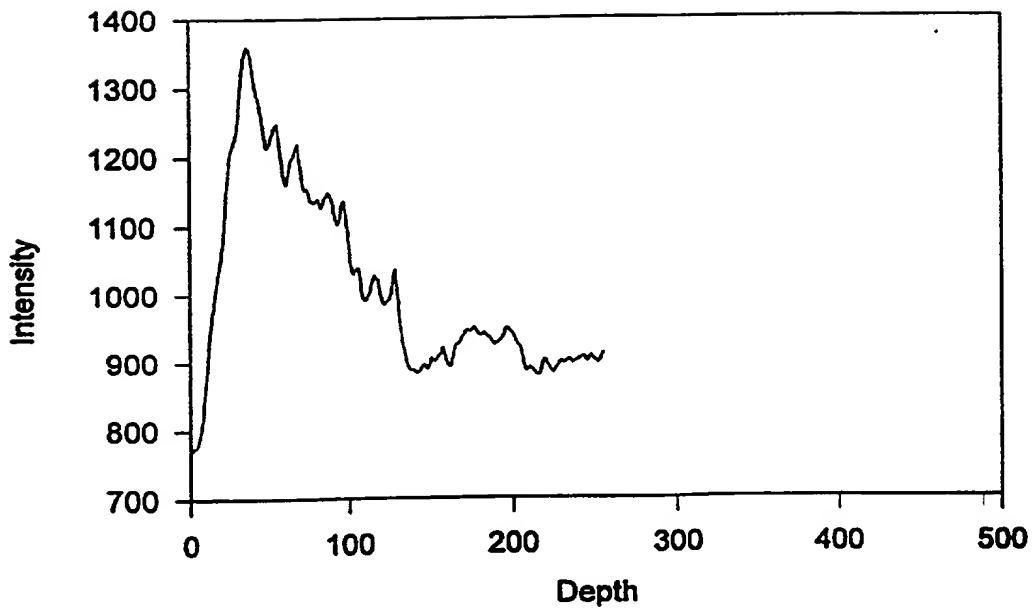


Figure 17

08/913257 (PCT/GB96/00553)

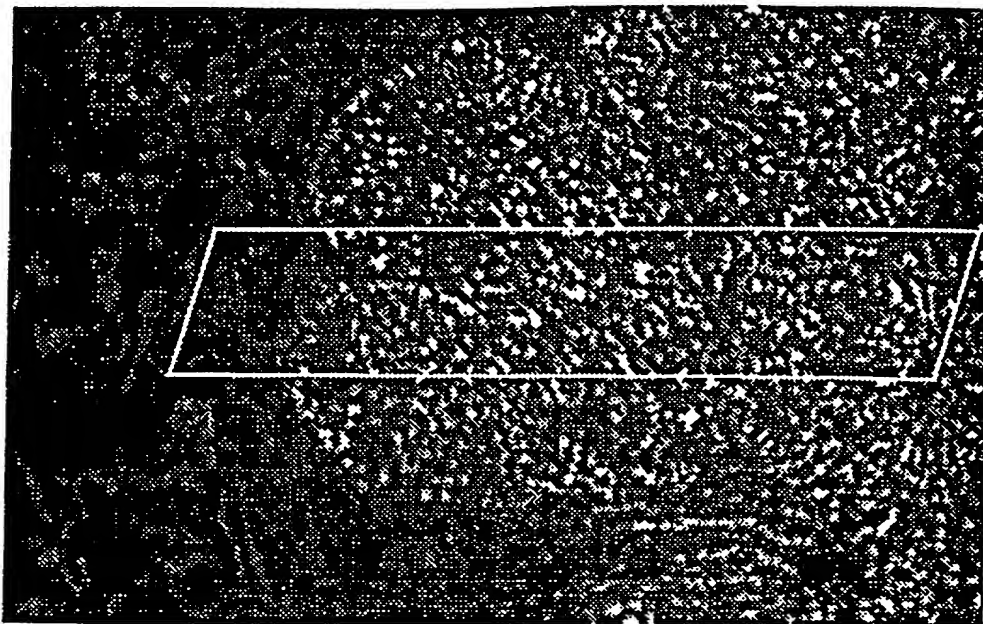
08/913257

PCT/GB96/00553

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APPROVED	O.G. FIG.
BY	CLASS SUBCLASS
DRAFTSMAN	WO 96/28412

B



161-6

A

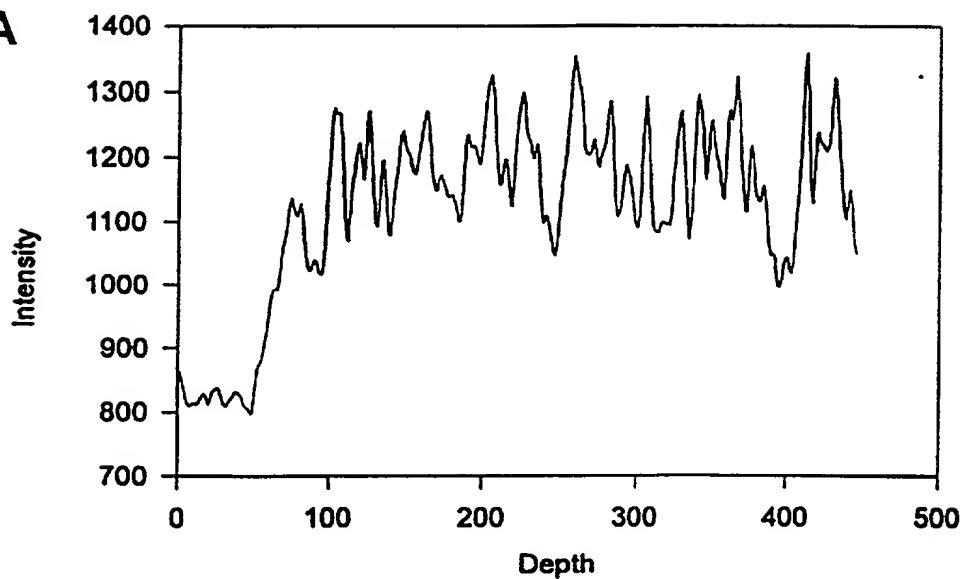


Figure 18

08/913257/521630

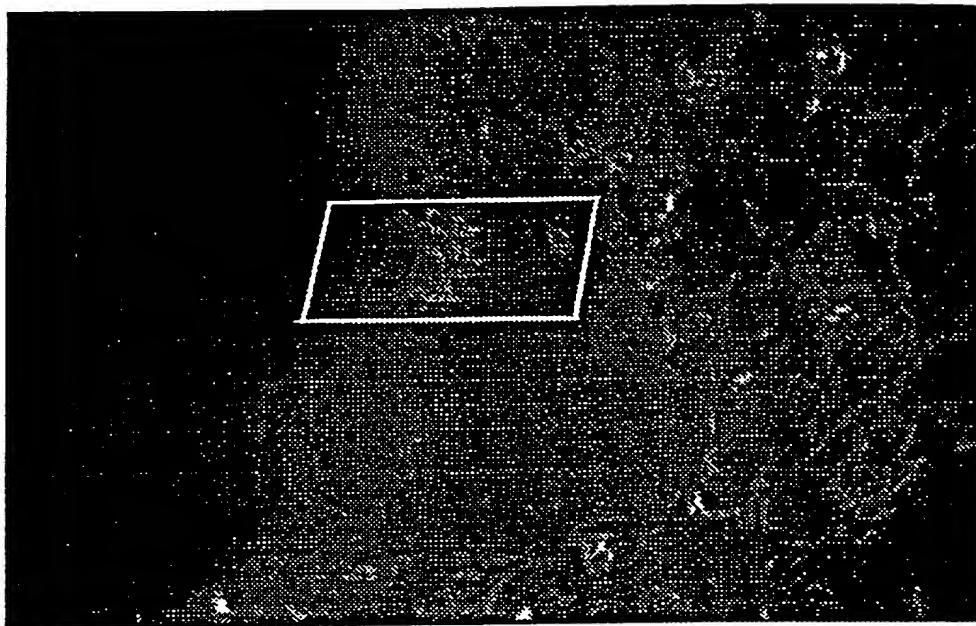
APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN	WO 96/28412	

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PCT/GB96/00553

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162-1

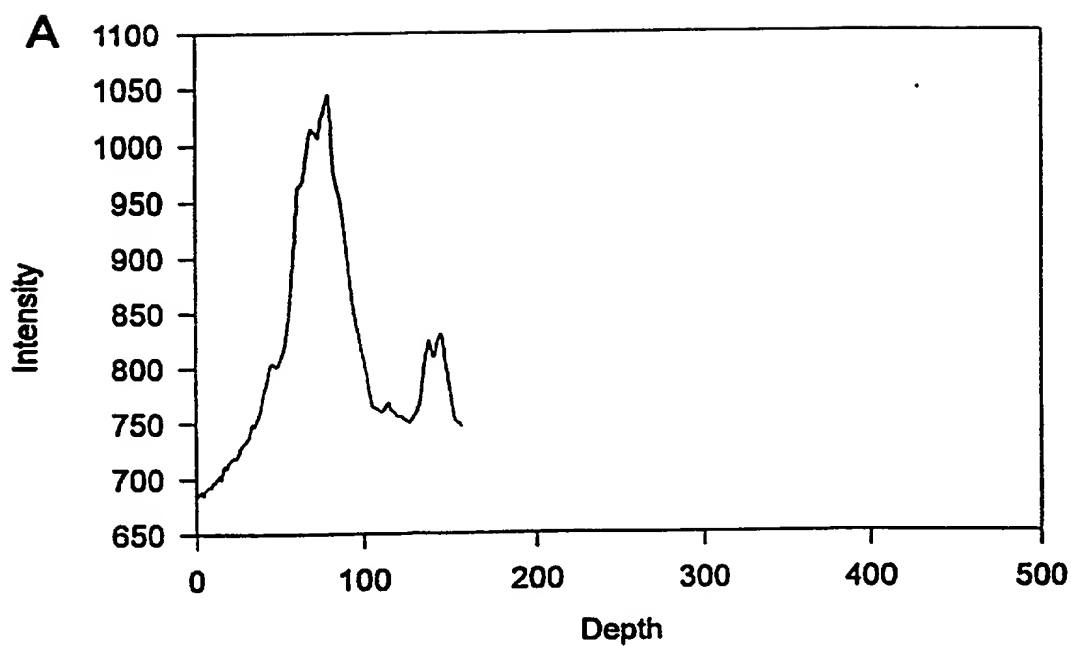
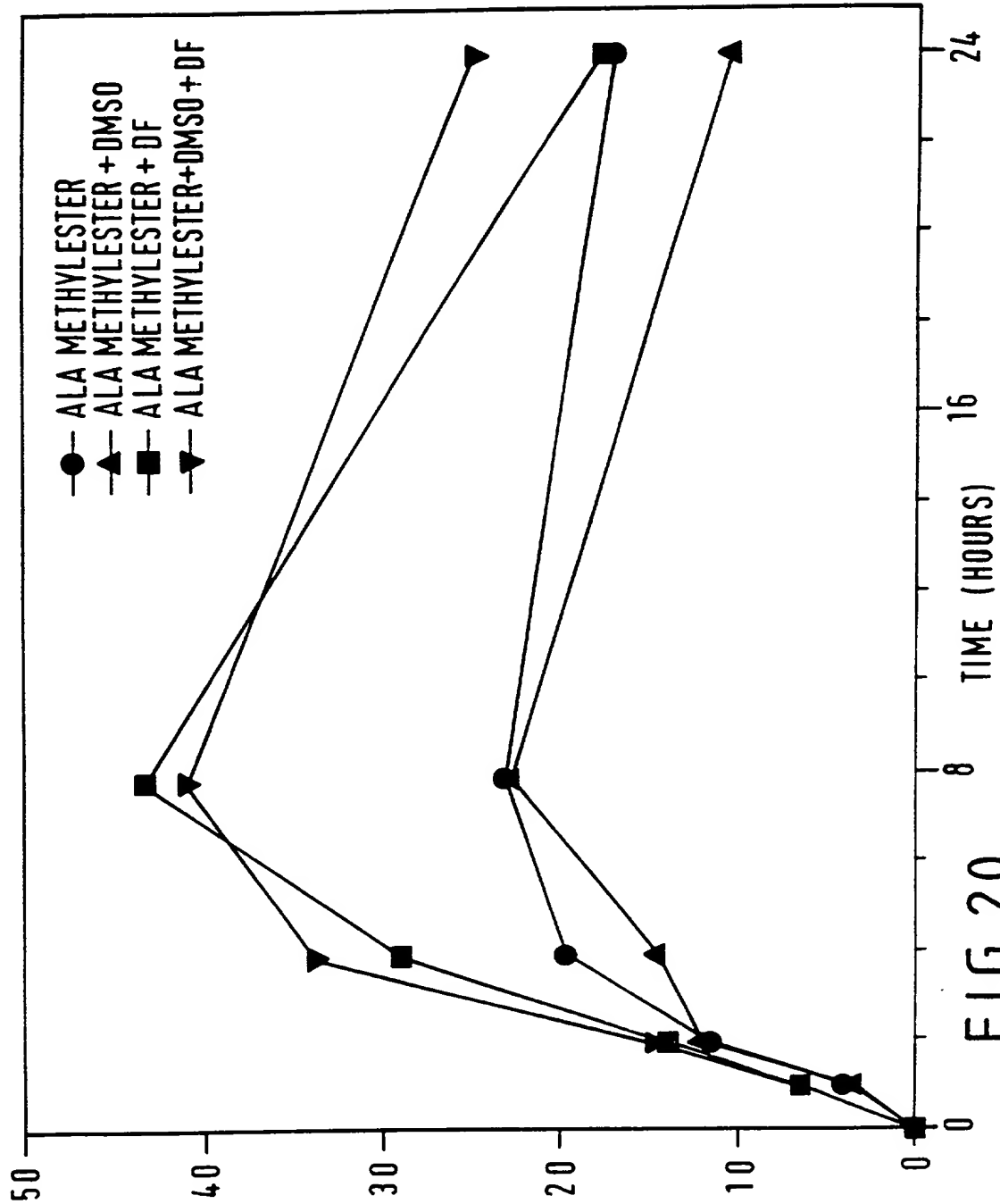


Figure 19

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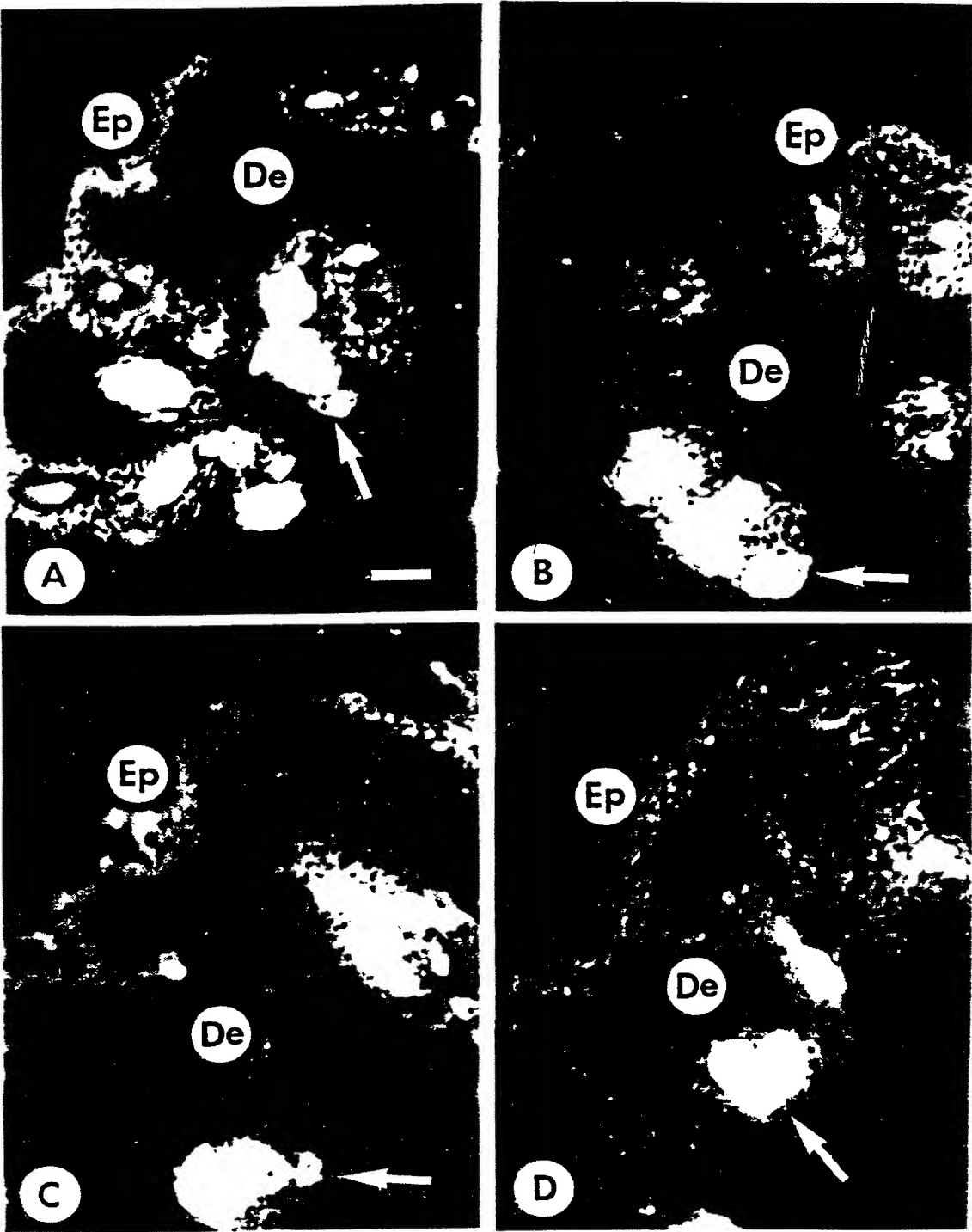
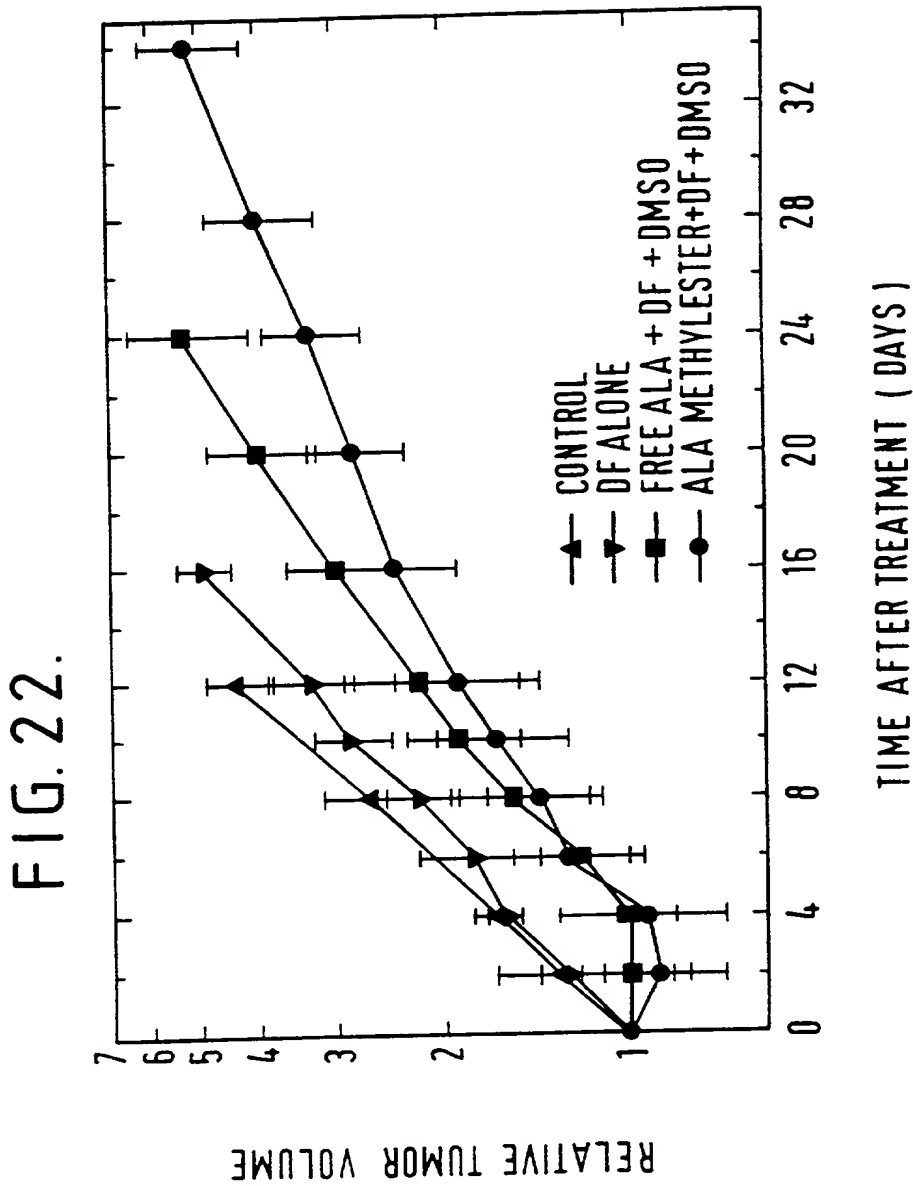


FIG. 21.

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APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN	WO 96/28412	

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PCT/GB96/00553

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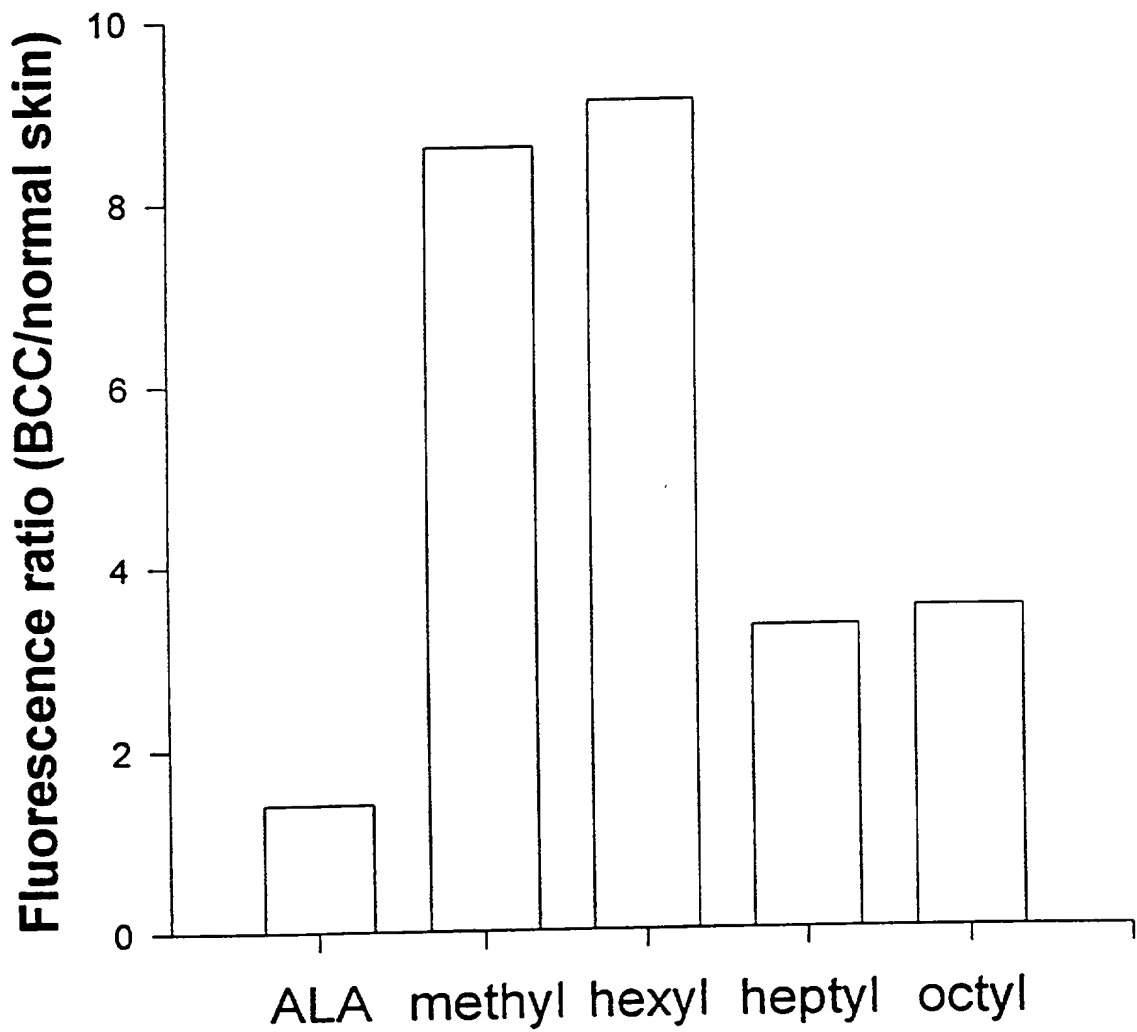


Figure 23

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: ESTERS OF 5-AMINOLEVULINIC ACID AS PHOTSENSITIZING AGENTS IN PHOTOCHEMOTHERAPY.

The specification of which was filed on September 10, 1997 as application serial no. 08/913,257 and was amended on September 10, 1997.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (see page 4 attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

Foreign application(s), if any, claiming priority under 35 U.S.C. § 119:

<u>Application Number</u>	<u>Country</u>	<u>Day/Month/Year Filed</u>
9504948.2	Great Britain	10/03/1995
9525822.4	Great Britain	18/12/1995

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

No such applications have been filed.

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

<u>Application Number</u>	<u>Filing Date</u>	<u>Status</u>
PCT/GB96/00553	March 8, 1996	Complete

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Anglin, J. Michael	Reg No 24,916	Embretson Janet E	Reg No 39,665	Litman, Mark A	Reg No 26,390
Bianchi, Timothy E	Reg No 39,610	Fogg David N	Reg No 35,138	Lundberg, Steven W	Reg No 30,568
Billig, Patrick G	Reg No 38,080	Forrest, Bradley A	Reg No 30,837	Madrid, Andres N	Reg No 40,710
Billion, Richard E	Reg No 32,836	Harris, Robert J	Reg No 37,346	Schwegman, Micheal L	Reg No 25,816
Brennan, Thomas F	Reg No 35,075	Hofmann, Rudolph P, Jr	Reg No 38,187	Simboli, Paul B	Reg No 38,616
Brooks, Edward J, III	Reg No 40,925	Holloway, Sheryl S	Reg No 37,850	Slifer, Russell D	Reg No 39,838
Clark, Barbara J	Reg No 38,107	Klima-Silberg, Catherine I	Reg No 40,052	Viksnins, Ann S	Reg No 37,748
Drake, Eduardo E	Reg No 40,594	Kluth, Daniel J	Reg No 32,146	Woessner Warren D	Reg No 30,440
Dryja, Michael A	Reg No 39,662	Lemaire, Charles A	Reg No 36,198		

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization/who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Schwegman, Lundberg, Woessner & Kluth, P.A. to the contrary.

Please direct all correspondence in this case to Schwegman, Lundberg, Woessner & Kluth, P.A. at the address indicated below:

P.O. Box 2938, Minneapolis, MN 55402
Telephone No. (612)373-6900

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of joint inventor number 1 : Karl E. Giersckey *HCO*

Citizenship: Norway

Residence: Oslo, Norway

Post Office Address: The Norwegian Radium Hospital
Montebello
Oslo N-0310
Norway

Signature: *Karl E. Giersckey*
Karl E. Giersckey

Date: 08.10.97

Full Name of joint inventor number 2 : Johan Moan *200*

Citizenship: Norway

Residence: Oslo, Norway

Post Office Address: Institute of Cancer Research
Montebello
Oslo N-0310
Norway

Signature: *Johan Moan*
Johan Moan

Date: 10.10.97

Full Name of joint inventor number 3 : Qian Peng *300*

Citizenship: Peoples Republic of China

Residence: Oslo, Norway

Post Office Address: Institute of Cancer Research
Montebello
Oslo N-0310
Norway

Signature: *Qian Peng*
Qian Peng

Date: 08-10-1997

Full Name of joint inventor number 4 : Harald Steen *400*

Citizenship: Norway

Residence: Oslo, Norway

Post Office Address: Institute of Cancer Research
Montebello
Oslo N-0310
Norway

Signature: *Harald Steen*
Harald Steen

Date: 9/10-97

☒ Additional inventors are being named on separately numbered sheets, attached hereto.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of joint inventor number 5 : Trond Warloe 500

Citizenship: Norway

Residence: Oslo, Norway

Post Office Address: The Norwegian Radium Hospital
Montebello
Oslo N-0310
Norway

Signature: Trond Warloe

Date: 08.10.97

Trond Warloe

Full Name of joint inventor number 6 : Alf Bjørseth 600

Citizenship: Norway

Residence: Oslo, Norway

Post Office Address: The Norwegian Radium Hospital
Research Foundation
Montebello
Oslo N-0310
Norway

Signature: Alf Bjørseth

Date: 07.10.97

Alf Bjørseth

Full Name of inventor:

Citizenship:

Residence:

Post Office Address:

Signature: _____

Date: _____

Full Name of inventor:

Citizenship:

Residence:

Post Office Address:

Signature: _____

Date: _____

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
 - (i) Opposing an argument of unpatentability relied on by the Office, or
 - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

- (1) Each inventor named in the application;
- (2) Each attorney or agent who prepares or prosecutes the application; and
- (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

SMALL BUSINESS

697.002US1


VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
 (37 C.F.R. 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

- a) ☐ the owner of the small business concern identified below:
- b) ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: PhotoCure AS
 ADDRESS OF CONCERN: Norveien 7
Montebello
Oslo N-0310 Norway

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 C.F.R. 121.3-18, and reproduced in 37 C.F.R. 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled ESTERS OF 5-AMINOLEVULINIC ACID AS PHOTSENSITIZING AGENTS IN PHOTOCHEMOTHERAPY by inventors Karl E. Giersckly, Johan Moan, Qian Peng, Harald Steen, Trond Warloe and Alf Bjorseth described in application serial no. 08/913,257, filed September 10, 1997

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 C.F.R. 1.9(c) or by any concern which would not qualify as a small business concern under 37 C.F.R. 1.9(d) or a nonprofit organization under 37 C.F.R. 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. 1.27)

NAME _____
 ADDRESS _____
 a) ☐ INDIVIDUAL b) ☐ SMALL BUSINESS CONCERN c) ☐ NONPROFIT ORGANIZATION

NAME _____
 ADDRESS _____
 a) ☐ INDIVIDUAL b) ☐ SMALL BUSINESS CONCERN c) ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereof, or any patent to which this verified statement is directed.

NAME Vidar Hansson M.D., Ph.D. PhotoCure AS
 TITLE/POSITION Professor, President P.O. Box 55, Montebello
0310 Oslo, Norway
 ADDRESS _____
 SIGNATURE DATE 6/10/97

FORM PTO-1390
(REV 10-96)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

697.002US1

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

08/913257

INTERNATIONAL APPLICATION NO.

PCT/GB96/00553

INTERNATIONAL FILING DATE

8 March 1996

PRIORITY DATE CLAIMED

10 March 1995

TITLE OF INVENTION : Esters of 5-Aminolevulinic Acid As Photosensitizing Agents in Photochemotherapy

APPLICANT(S) FOR DO/EO/US : Karl E. Giersckey et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

Copies of Request, International Search Report, International Application as published, and International Preliminary Examination Report.

PCT/GB96/00553

697.002US1

17. ☒ The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :**

Search Report has been prepared by the EPO or JPO \$910.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
..... \$700.00No international preliminary examination fee paid to USPTO (37 CFR 1.482)
but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$770.00Neither international preliminary examination fee (37 CFR 1.482) nor
international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$1040.00International preliminary examination fee paid to USPTO (37 CFR 1.482)
and all claims satisfied provisions of PCT Article 33(2)-(4) \$96.00**ENTER APPROPRIATE BASIC FEE AMOUNT =****CALCULATIONS** PTO USE ONLY

\$ 910.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$ 130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	29 - 20 =	9	X \$22.00
Independent claims	2 - 3 =	0	X \$80.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00

\$ 198.00

\$ 0

260.00

TOTAL OF ABOVE CALCULATIONS =

\$ 1498.00

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement
must also be filed (Note 37 CFR 1.9, 1.27, 1.28).

\$

SUBTOTAL =

\$ 1498.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

+

TOTAL NATIONAL FEE =

\$ 1498.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property

\$

+

TOTAL FEES ENCLOSED =

\$ 1498.00

Amount to be:
refunded

\$

charged

\$

a. ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.b. ☒ Please charge my Deposit Account No. 19-0743 in the amount of \$ 1498.00 to cover the above fees.
A duplicate copy of this sheet is enclosed.c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
overpayment to Deposit Account No. 19-0743. A duplicate copy of this sheet is enclosed.**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO

Ann S. Viksnins
Schwegman, Lundberg, Woessner & Kluth, P.A.
P.O. Box 2938
Minneapolis, MN 55402

SIGNATURE

Ann S. Viksnins

NAME

37,748

REGISTRATION NUMBER

INTERNATIONAL SEARCH REPORT

International Application No
PC./GB 96/00553

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07C229/22 C07C227/18 A61K31/195

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"Database Xfire, entries 3060978, 5347132, 5499790, 5620924, 5633390, 5991317 and 6517740", BEILSTEIN XP002005286	1-6
X	--- PATENT ABSTRACTS OF JAPAN vol. 016, no. 156 (C-0930), 16 April 1992 & JP,A,04 009360 (COSMO SOGO KENKYUSHO:KK;OTHERS: 01), 14 January 1992, see abstract	1-6
X	--- EP,A,0 316 179 (TOKUYAMA SODA KK) 17 May 1989 see Table I-A, line 41 (p. 44) --- -/--	1-6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search

11 June 1996

Date of mailing of the international search report

28.05.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

Janus, S

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB96/00553

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 14 and 15 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

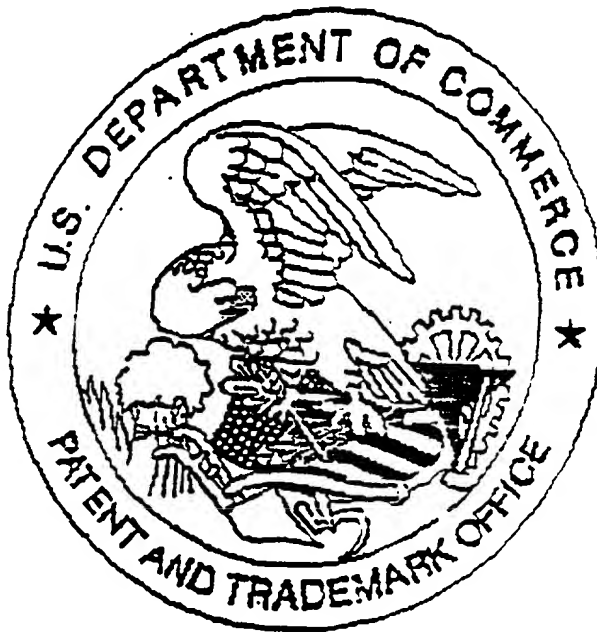
International Application No
PCT/GB 96/00553

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-2411382		SE-A- 7502592	10-09-75
		US-A- 4087532	02-05-78

WO-A-9101727	21-02-91	US-A- 5079262	07-01-92
		AU-B- 624985	25-06-92
		AU-B- 6034390	11-03-91
		JP-T- 4500770	13-02-92
		NL-T- 9021172	01-07-91
		US-A- 5422093	06-06-95
		US-A- 5211938	18-05-93
		US-A- 5234940	10-08-93

United States Patent & Trademark Office

Office of Initial Patent Examination – Scanning Division



Application deficiencies found during scanning:

1. Application papers are not suitable for scanning and are not in compliance with 37 CFR 1.52 because:

- ☐ All sheets must be the same size and either A4 (21 cm x 29.7 cm) or 8-1/2" x 11". Pages _____ do not meet these requirements.
- ☐ Papers are not flexible, strong, smooth, non-shiny, durable, and white.
- ☐ Papers are not typewritten or mechanically printed in permanent ink on one side.
- ☐ Papers contain improper margins. Each sheet must have a left margin of at least 2.5 cm (1") and top, bottom and right margins of at least 2.0 cm (3/4").
- ☐ Papers contain hand lettering.

2. Drawings are not in compliance and were not scanned because:

- ☐ The drawings or copy of drawings are not suitable for electronic reproduction.
- ☐ All drawings sheets are not the same size. Pages must be either A4 (21 cm x 29.7 cm) or 8-1/2" x 11".
- ☐ Each sheet must include a top and left margin of at least 2.5 cm (1"), a right margin of at least 1.5 cm (9/16") and a bottom margin of at least 1.0 cm (3/8").

3. Page(s) _____ are not of sufficient clarity, contrast and quality for electronic reproduction.

4. Page(s) _____ are missing.

5. OTHER: no transmittal

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